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Patentanmeldung Nr. Patent application No. Demande de brevet n°

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Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Anmelder: Applicant(s): Demandeur(s):

Introgene B.V.
NL-2288 GJ Rijswijk
NETHERLANDS
Rijksuniversiteit te Leiden
2312 AV Leiden
NETHERLANDS

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Title: **Improved materials derived from recombinant adenovirus to be used in gene therapy.**

The invention relates to the field of recombinant DNA technology, more in specifically to the field of gene therapy. In particular the invention relates to gene therapy using materials derived from adenovirus, in particular human recombinant adenovirus.It especially relates to novel virus derived vectors and novel packaging cell lines for vectors based on adenoviruses.

Gene therapy is a recently developed concept for which a wide range of applications can be and have been envisaged.

In gene therapy a molecule carrying genetic information is introduced into some or all cells of a host, as a result of which the genetic information is added to the host in a functional format.

The genetic information added may be a gene or a derivative of a gene, such as a cDNA, which encodes a protein. In this case the functional format means that the protein can be expressed by the machinery of the host cell.

The genetic information can also be a sequence of nucleotides complementary to a sequence of nucleotides (be it DNA or RNA) present in the host cell. The functional format in this case is that the added DNA (nucleic acid) molecule or copies made thereof <u>in situ</u> are capable of base pairing with the complementary sequence present in the host cell.

Applications include the treatment of genetic disorders by supplementing a protein or other substance which is, through said genetic disorder, not present or at least present in insufficient amounts in the host, the treatment of tumors and (other) acquired diseases such as (auto)immune diseases or infections, etc.

As may be clear from the above, there are basically three different approaches in gene therapy, one directed towards compensating a deficiency present in a (mammalian) host; the second directed towards the removal or elimination of unwanted

substances (organisms or cells) and the third towards application of a recombinant vaccine (tumors or foreign micro-organisms).

For the purpose of gene therapy, adenoviruses carrying deletions have been proposed as suitable vehicles.

Adenoviruses are non-enveloped DNA viruses. Gene-transfer vectors derived from adenoviruses (so-called adenoviral vectors) have a number of features that make them particularly useful for gene transfer for such purposes. Eg. the biology of the adenoviruses is characterized in detail, the adenovirus is not associated with severe human pathology, the virus is extremely efficient in introducing its DNA into the host cell, the virus can infect a wide variety of cells and has a broad host-range, the virus can be produced in large quantities with relative ease, and the virus can be rendered replication defective by deletions in the early-region 1 (E1) of the viral genome.

During the productive infection cycle, the viral genes are expressed in two phases: the early phase, which is the period upto viral DNA replication, and the late phase, which coincides with the initiation of viral DNA replication. During the early phase only the early gene products, encoded by regions E1, E2, E3 and E4, are expressed, which carry out a number of functions that prepare the cell for synthesis of viral structural proteins (Berk, 1986). During the late phase the late viral gene products are expressed in addition to the early gene products and host cell DNA and protein synthesis are shut off. Consequently, the cell becomes dedicated to the production of viral DNA and of viral structural proteins (Tooze, 1981).

The E1 region of adenovirus is the first region of adenovirus expressed after infection of the target cell. This region consists of two transcriptional units, the E1A and E1B genes, which both are required for oncogenic transformation of primary (embryonal) rodent cultures. The main functions of the E1A gene products are i) to induce quiescent cells to enter

the cell cycle and resume cellular DNA synthesis, and ii) to transcriptionally activate the E1B gene and the other early regions (E2, E3, E4). Transfection of primary cells with the E1A gene alone can induce unlimited proliferation (immortalization), but does not result in complete transformation. However, expression of E1A in most cases results in induction of programmed cell death (apoptosis), and only occasionally immortalization is obtained (Jochemsen *et al.*, 1987). Co-expression of the E1B gene is required to prevent induction of apoptosis and for complete morphological transformation to occur. In established immortal cell lines, high level expression of E1A can cause complete transformation in the absence of E1B (Roberts *et al.*, 1985).

The E1B encoded proteins assist E1A in redirecting the cellular functions to allow viral replication. The E1B 55 kD and E4 33kD proteins, which form a complex that is essentially localized in the nucleus, function in inhibiting the synthesis of host proteins and in facilitating the expression of viral genes. Their main influence is to establish selective transport of viral mRNAs from the nucleus to the cytoplasm, concomittantly with the onset of the late phase of infection. The E1B 21 kD protein is important for correct temporal control of the productive infection cycle, thereby preventing premature death of the host cell before the virus life cycle has been completed. Mutant viruses incapable of expressing the E1B 21 kD gene-product exhibit a shortened infection cycle that is accompanied by excessive degradation of host cell chromosomal DNA (*deg*-phenotype) and in an enhanced cytopathic effect (*cyt*-phenotype) (Telling *et al.*, 1994). The *deg* and *cyt* phenotypes are suppressed when in addition the E1A gene is mutated, indicating that these phenotypes are a function of E1A (White *et al.*, 1988). Furthermore, the E1B 21 kDa protein slows down the rate by which E1A switches on the other viral genes. It is not yet known through which mechanism(s) E1B 21 kD quenches these E1A dependent functions.

Vectors derived from human adenoviruses, in which at least the E1 region has been deleted and replaced by a gene of interest, have been used extensively for gene therapy experiments in the pre-clinical and clinical phase.

The adenovirus genome is a linear double-stranded DNA molecule of approximately 36000 base pairs with the 55-kDa terminal protein covalently bound to the 5'terminus of each strand. The Ad DNA contains identical Inverted Terminal Repeats (ITR) of about 100 base pairs with the exact length depending on the serotype. The viral origins of replication are located within the ITRs exactly at the genome ends. DNA synthesis occurs in two stages. First, the replication proceeds by strand displacement, generating a daughter duplex molecule and a parental displaced strand. The displaced strandis single stranded and can form a so-called "panhandle" intermediate, which allows replication initiation and generation of a daughter duplex molecule. Alternatively, replication may proceed from both ends of the genome simultaneously, obviating the requirement to form the panhandle structure. The replication is summarized in Figure 11 adapted from (Lechner and Kelly, 1977).

As stated before all adenovirus vectors currently used in gene therapy have a deletion in the E1 region, where novel genetic information can be introduced. The E1 deletion renders the recombinant virus replication defective (Stratford-Perricaudet and Perricaudet, 1991). We have demonstrated that recombinant adenoviruses are able to efficiently transfer recombinant genes to the rat liver and airway epithelium of rhesus monkeys (Bout *et al.*, 1994b; Bout *et al.*, 1994a). In addition, we (Vincent *et al.*, 1995) and others (Haddada *et al.*, 1993) have observed a very efficient *in vivo* adenovirus mediated gene transfer to a variety of tumor cells *in vitro* and to solid tumors in animals models (lung tumors, glioma) and human xenografts in immunodeficient mice (lung) *in vivo*.

In contrast to for instance retroviruses, adenoviruses a) do not integrate into the host cell genome; b) are able to

infect non-dividing cells and c) are able to efficiently transfer recombinant genes *in vivo* (Brody and Crystal, 1994). Those features make adenoviruses attractive candidates for *in vivo* gene transfer of, for instance, suicide or cytokine genes into tumor cells.

However, a problem associated with current recombinant adenovirus technology is the possibility of unwanted generation of replication competent adenovirus (RCA) during the production of recombinant adenovirus (Lochmüller *et al.*, 1994). This is caused by homologous recombination between overlapping sequences from the recombinant vector and the adenovirus constructs present in the complementing cell line, such as the 293 cells (Graham *et al.*, 1977). RCA in batches to be used in clinical trials is unwanted because RCA i) will replicate in an uncontrolled fashion; ii) can complement replication defective recombinant adenovirus, causing uncontrolled multiplication of the recombinant adenovirus and iii) batches containing RCA induce significant tissue damage and hence strong pathological side effects (Lochmüller *et al.*, 1994). Therefore, batches to be used in clinical trials should be proven free of RCA (Ostrove, 1994). In one aspect of the invention this problem in virus production is solved in that we have developed packaging cells that have no overlapping sequences with a new basic vector and thus are suited for safe large scale production of recombinant adenoviruses.

One of the additional problems associated with the use of recombinant adenovirus vectors is the host-defence reaction against treatment with adenovirus.

Briefly, recombinant adenoviruses are deleted for the E1 region (see above). The adenovirus E1 products trigger the transcription of the other early genes (E2, E3, E4), which consequently activate expression of the late virus genes. Therefore, it was generally thought that E1 deleted vectors would not express any other adenovirus genes. However, recently it has been demonstrated that some cell types are able to express adenovirus genes in the absence of E1

sequences. This indicates, that some cell types possess the machinery to drive transcription of adenovirus genes. In particular, it was demonstrated that such cells synthesize E2A and late adenovirus proteins.

In a gene therapy setting, this means that transfer of the therapeutic recombinant gene to somatic cells not only results in expression of the therapeutic protein but may also result in the synthesis of viral proteins. Cells that express adenoviral proteins are recognized and killed by Cytotoxic T Lymphocytes, which thus a) eradicates the transduced cells and b) causes inflammations (Bout *et al.*, 1994a; Engelhardt *et al.*, 1993; Simon *et al.*, 1993). As this adverse reaction is hampering gene therapy, several solutions to this problem have been suggested, such as a) using immunosuppressive agents after treatment; b) retainment of the adenovirus E3 region in the recombinant vector (see patent application) and c) and using ts mutants of human adenovirus, which have a point mutation in the E2A region (patent WO/28938).

However, these strategies to circumvent the immune response have their drawbacks.

The use of ts mutant recombinant adenovirus diminishes the immune response to some extent, but was less effective in preventing pathological responses in the lungs (Engelhardt *et al.*, 1994a).

The E2A protein may induce an immune response by itself and it plays a pivotal role in the switch to the synthesis of late adenovirus proteins. Therefore, it is attractive to make recombinant adenoviruses which are mutated in the E2 region, rendering it temperature sensitive (ts), as has been claimed in patent application WO/28938.

A major drawback of this system is the fact that, although the E2 protein is unstable at the non-permissive temperature, the immunogenic protein is still being synthesized. In addition, it is to be expected that the unstable protein does activate late gene expression, albeit to a low extent. ts125 mutant recombinant adenoviruses have been

tested, and prolonged recombinant gene expression was reported (Yang *et al.*, 1994b; Engelhardt *et al.*, 1994a; Engelhardt *et al.*, 1994b; Yang *et al.*, 1995). However, pathology in the lungs of cotton rats was still high (Engelhardt *et al.*, 1994a), indicating that the use of ts mutants results in only a partial improvement in recombinant adenovirus technology. An additional difficulty associated with the use of ts125 mutant adenoviruses is that a high frequency of reversion is observed. These revertants are either real revertants or the result of second site mutations (Kruijer *et al.*, 1983; Nicolas *et al.*, 1981). Both types of revertants have an E2A protein that functions at normal temperature and have therefore similar toxicity as the wild-type virus.

In another aspect of the present invention we therefore delete E2A coding sequences from the recombinant adenovirus genome and transfect these E2A sequences into the (packaging) cell lines containing E1 sequences to complement recombinant adenovirus vectors.

Major hurdles in this approach are a) that E2A should be expressed to very high levels and b) that E2A protein is very toxic to cells.

The current invention in yet another aspect therefore discloses use of the ts125 mutant E2A gene, which produces a protein that is not able to bind DNA sequences at the non permissive temperature. High levels of this protein may be maintained in the cells (because it is not toxic at this temperature) until the switch to the permissive temperature is made. This can be combined with placing the mutant E2A gene under the direction of an inducible promoter, such as for instance tet, methallothionein, steroid inducible promoter, retinoic acid ß-receptor or other inducible systems. However in yet another aspect of the invention, the use of an inducible promoter to control the moment of production of toxic wild-type E2A is disclosed.

Two salient additional advantages of E2A-deleted recombinant adenovirus are the increased capacity to harbor

heterologous sequences and the permanent selection for cells that express the mutant E2A. This second advantage relates to the high frequency of reversion of ts125 mutation: when reversion occurs in a cell line harboring ts125 E2A, this will be lethal to the cell. Therefore, there is a permanent selection for those cells that express the ts125 mutant E2A protein. In addition, as we in one aspect of the invention generate E2A-deleted recombinant adenovirus, we will not have the problem of reversion in our adenoviruses.

In yet another aspect of the invention as a further improvement the use of non-human cell lines as packaging cell lines is disclosed.

For GMP production of clinical batches of recombinant viruses it is desirable to use a cell line that has been used widely for production of other biotechnology products. For instance cells of monkey origin have been used e.g. vaccines to produce. These cells can not be used directly for the production of recombinant human adenovirus, as human adenovirus can not or only to low levels replicate in cells of monkey origin. A block in the switch of early to late phase of adenovirus lytic cycle is underlying defective replication. However, host range mutations in the human adenovirus genome are described (hr400 - 404) which allow replication of human viruses in monkey cells. These mutations reside in the gene encoding E2A protein (Klessig and Grodzicker, 1979; Klessig *et al.*, 1984; Rice and Klessig, 1985)(Klessig *et al.*, 1984). Moreover, mutant viruses have been described that harbor both the hr and temperature-sensitive ts125 phenotype (Brough *et al.*, 1985; Rice and Klessig, 1985).

We therefore generate packaging cell lines of monkey origin (e.g. VERO, CV1) that harbor:

a. E1 sequences, to allow replication of E1/E2 defective adenoviruses, and
b. E2A sequences, containing the hr mutation and the ts 125 mutation, named ts400 (Brough *et al.*, 1985; Rice and

Klessig, 1985) to prevent cell death by E2A overexpression, and/or

c. E2A sequences, just containing the hr mutation, under the control of an inducible promoter, and/or

d. E2A sequences, containing the hr mutation and the ts 125 mutation (ts400), under the control of an inducible promoter

Furthermore we disclose the construction of novel and improved combinations of (novel and improved) packaging cell lines and (novel and improved) recombinant adenovirus vectors. We provide:

1. a novel packaging cell line derived from diploid human embryonic retinoblasts (HER) that harbors nt. 80 - 5788 of the Ad5 genome. This cell line, named 911, deposited under no 95062101 at the ECACC, has many characteristics that make it superior to the commonly used 293 cells.
2. novel packaging cell lines that express just E1A genes and not E1B genes.

Established cell lines (and not human diploid cells of which 293 and 911 cells are derived) are able to express E1A to high levels without undergoing apoptotic cell death, as occurs in human diploid cells that express E1A in the absence of E1B.

Such cell lines are able to trans-complement E1B-defective recombinant adenoviruses, because viruses mutated for E1B 21 kD protein are able to complete viral replication even faster than wild-type adenoviruses (Telling *et al.*, 1994). The constructs are described in detail below, and graphically represented in Figures 1-5. The constructs are transfected into the different established cell lines and are selected for high expression of E1A. This is done by operatively linking a selectable marker gene (e.g. NEO gene) directly to the E1B promoter. The E1B promoter is transcriptionally activated by the E1A gene product and therefore resistance to the selective agent (e.g. G418 in

the case NEO is used as the selection marker) results in direct selection for desired expression of the E1A gene.

3. Packaging constructs that are mutated or deleted for E1B 21 kD, but just express the 55 kD protein.

4. Packaging constructs to be used for generation of complementing packaging cell lines from diploid cells (not exclusively of human origin) do not need selection with marker genes. These cells are immortalized by expression of E1A. However, in this particular case expression of E1B is essential to prevent apoptosis induced by E1A proteins. Selection of E1 expressing cells is achieved by selection for focus formation (immortalization), as described for 293 cells (Graham *et al.*, 1977) and 911 cells (see 1), that are E1-transformed human embryonic kidney (HEK) and human embryonic retinoblasts (HER), respectively.

5. New adenovirus vectors with extended E1 deletions (E1 deletion nt. 459 - 3510). These viral vectors lack sequence overlap with the E1 complementing sequences in said packaging cell lines. These vectors preferably do contain pIX promoter sequences.

6. E2A expressing packaging cell lines preferably based on either E1A expressing established cell lines or E1A + E1B expressing diploid cells (see under 2 - 4). E2A expression is either under the control of an inducible promoter or the E2A ts125 mutant is driven by either an inducible or a constitutive promoter.

7. Recombinant adenovirus vectors as described before (see 5) but carrying an additional deletion of E2A sequences.

8. Adenovirus packaging cells from monkey origin that are able to trans-complement E1-defective recombinant adenoviruses. They are preferably co-transfected with pIG.E1AE1B and pIG.NEO, and selected for NEO resistance. Such cells expressing E1A and E1B are able to trans-complement E1 defective recombinant human adenoviruses, but will do so inefficiently because of a block of the synthesis of late adenovirus proteins in cells of monkey

origin (Klessig and Grodzicker, 1979). To overcome this problem, we generate recombinant adenoviruses that harbor a host-range mutation in the E2A gene, allowing human adenoviruses to replicate in monkey cells. Such viruses are generated as described in Figure 8, except DNA from a hr- mutant is used for homologous recombination.

9. Adenovirus packaging cells from monkey origin as described under 8, except that they will also be co-transfected with E2A sequences harboring the hr mutation. This allows replication of human adenoviruses lacking E1 and E2A (see under 7). E2A in these cell lines is either under the control of an inducible promoter or the tsE2A mutant is used. In the latter case, the E2A gene will thus carry both the ts mutation and the hr mutation (derived from ts400). Replication competent human adenoviruses have been described that harbor both mutations (Brough *et al.*, 1985; Rice and Klessig, 1985).

A further aspect of the invention provides otherwise improved adenovirus vectors, as well as novel strategies for generation and application of such vectors and a method for the intracellular amplification of linear DNA fragments in mammalian cells.

The so-called "minimal" adenovirus vectors according to the present invention retain at least a portion of the viral genome that is required for encapsidation of the genome into virus particles (the encapsidation signal), as well as at least one copy of at least a functional part or a derivative of the Inverted Terminal Repeat (ITR), that is DNA sequences derived from the termini of the linear adenovirus genome. The vectors according to the present invention will also contain a transgene linked to a promoter sequence to govern expression of the transgene. Packaging of the so-called minimal adenovirus vector can be achieved by co-infection with a helper virus or, alternatively, with a packaging deficient replicating helper system as described below.

Adenovirus-derived DNA fragments that can replicate in suitable cell lines and that may serve as a packaging deficient replicating helper system are generated as follows. These DNA fragments retain at least a portion of the transcribed region of the "late" transcription unit of the adenovirus genome and carry deletions in at least a portion of the E1 region and deletions in at least a portion of the encapsidation signal. In addition, these DNA fragments contain at least one copy of an inverted terminal repeat (ITR). At one terminus of the transfected DNA molecule an ITR is located. The other end may contain an ITR, or alternatively, a DNA sequence that is complementary to a portion of the same strand of the DNA molecule other than the ITR. If, in the latter case, the two complementary sequences anneal, the free 3'-hydroxyl group of the 3' terminal nucleotide of the hairpin-structure can serve as a primer for DNA synthesis by cellular and/or adenovirus-encoded DNA polymerases, resulting in conversion into a double-stranded form of at least a portion of the DNA molecule. Further replication initiating at the ITR will result in a linear double-stranded DNA molecule, that is flanked by two ITR's, and is larger than the original transfected DNA molecule (see Fig.10). This molecule can replicate itself in the transfected cell by virtue of the adenovirus proteins encoded by the DNA molecule and the adenoviral and cellular proteins encoded by genes in the host-cell genome. This DNA molecule can not be encapsidated due to its large size (greater than 39000 base pairs) or due to the absence of a functional encapsidation signal. This DNA molecule is intended to serve as a helper for the production of defective adenovirus vectors in suitable cell lines.

The invention also comprises a method for the amplification of linear DNA fragments of variable size in suitable mammalian cells. These DNA fragments contain at least one copy of the ITR at one of the termini of the fragment. The other end may contain an ITR, or alternatively, a DNA sequence that is complementary to a portion of the same strand of the

DNA molecule other than the ITR. If, in the latter case, the two complementary sequences anneal, the free 3'-hydroxyl group of the 3' terminal nucleotide of the hairpin-structure can serve as a primer for DNA synthesis by cellular and/or adenovirus-encoded DNA polymerases, resulting in conversion of the displaced stand into a double stranded form of at least a portion of the DNA molecule. Further replication initiating at the ITR will result in a linear double-stranded DNA molecule, that is flanked by two ITR's, which is larger than the original transfected DNA molecule. A DNA molecule that contains ITR sequences at both ends can replicate itself in transfected cells by virtue of the presence of at least the adenovirus E2 proteins (viz. the DNA-binding protein (DBP), the adenovirus DNA polymerase (Ad-pol), and the preterminal protein (pTP). The required proteins may be expressed from adenovirus genes on the DNA molecule itself, from adenovirus E2 genes integrated in the host-cell genome, or from a replicating helper fragment as described above.

Several groups have shown that the presence of ITR sequences at the end of DNA molecules are sufficient to generate adenovirus minichromosomes that can replicate, if the adenovirus-proteins required for replication are provided in trans e.g. by infection with a helpervirus (Hu *et al.*, 1992); (Wang and Pearson, 1985); (Hay *et al.*, 1984). Hu *et al.*, (1992) observed the presence and replication of symmetrical adenovirus minichromosome-dimers after transfection of plasmids containing a single ITR. The authors were able to demonstrate that these dimeric minichromosomes arize after tail-to-tail ligation of the single ITR DNA molecules. In DNA extracted from defective adenovirus type 2 particles, dimeric molecules of various sizes have also been observed using electron-microscopy (Daniell, 1976). It was suggested that the incomplete genomes were formed by illegitimate recombination between different molecules and that variations in the position of the sequence at which the illegitimate base pairing occurred were resonsible for the heterogeneous nature

of the incomplete genomes. Based on this mechanism it was speculated that, in theory, defective molecules with a total length of up to two times the normal genome could be generated. Such molecules could contain duplicated sequences from either end of the genome. However, no DNA molecules larger than the full-length virus were found packaged in the defective particles (Daniell, 1976). This can be explained by the size-limitations that apply to the packaging. In addition, it was observed that in the virus particles DNA-molecules with a duplicated left-end predominated over those containing the right-end terminus (Daniell, 1976). This is fully explained by the presence of the encapsidation signal near that left-end of the genome (Gräble and Hearing, 1990; Gräble and Hearing, 1992; Hearing *et al.*, 1987).

The major problems associated with the current adenovirus-derived vectors are:

A) The strong immunogenicity of the virus particle

B) The expression of adenovirus genes that reside in the adenoviral vectors, resulting in a Cytotoxic T-cell response against the transduced cells.

C) The low amount of heterologous sequences that can be accommodated in the current vectors (Up to maximally approx. 8000 bp. of heterologous DNA).

Ad A) The strong immunogenicity of the adenovirus particle results in an immunological response of the host, even after a single administration of the adenoviral vector. As a result of the development of neutralizing antibodies, a subsequent administration of the virus will be less effective or even completely ineffective. However, a prolonged or persistent expression of the transferred genes will reduce the number of administrations required and may bypass the problem.

Ad B) Experiments performed by Wilson and collaborators have demonstrated that after adenovirus-mediated gene transfer into immunocompetent animals, the expression of the transgene gradually decreases and disappears approximately 2- 4 weeks

post-infection (Yang *et al.*, 1994a; Yang *et al.*, 1994b). This is caused by the development of a Cytotoxic T-Cell (CTL) response against the transduced cells. The CTLs were directed against adenovirus proteins expressed by the viral vectors. In the transduced cells synthesis of the adenovirus DNA-binding protein (the E2A-gene product), penton and fiber proteins (late-gene products) could be established. These adenovirus proteins, encoded by the viral vector, were expressed despite deletion of the E1 region. This demonstrates that deletion of the E1 region is not sufficient to completely prevent expression of the viral genes (Engelhardt *et al.*, 1994a).

Ad C) Studies by Graham and collaborators have demonstrated that adenoviruses are capable of encapsidating DNA of up to 105% of the normal genome size (Bett *et al.*, 1993). Larger genomes tend to be instable resulting in loss of DNA sequences during propagation of the virus. Combining deletions in the E1 and E3 regions of the virual genomes increases the maximmum size of the foreign that can be encapsidated to approx. 8.3 kb. In addition, some sequences of the E4 region appear to be dispensable for virus growth (adding another 1.8 kb to the maximum encapsidation capacity). Also the E2A region can be deleted from the vector, when the E2A gene product is provided in trans in the encapsidation cell line, adding another 1.6 kb. It is, however, unlikely that the maximum capacity of foreign DNA can be significantly increased further than 12 kb.

We developed a new strategy for the generation and production of helperfree-stocks of recombinant adenovirus vectors that can accomodate up to 38 kb of foreign DNA. Only two functional ITR sequences, and sequences that can function as an encapsidation signal need to be part of the vector genome. Such vectors are called minimal adenovectors. The helper functions for the minimal adenovectors are provided in trans by encapsidation defective-replication competent DNA molecules that contain all the viral genes encoding the required gene productes, with the exception of those genes

that are present in the host-cell genome, or genes that reside in the vector genome.

The invention will be illustrated in the following experimental part, which is only intended for said purpose and should not be used to reduce the scope of the present invention as understood by the person skilled in the art.

Use of the IG packaging constructs

Diploid cells.

The constructs, in particular pIG.E1A.E1B, will be used to transfect diploid human cells, such as Human Embryonic Retinoblasts (HER), Human Embryonic Kidney cells (HEK), and Human Embryonic Lung cells (HEL). Transfected cells will be selected for transformed phenotype (focus formation) and tested for their ability to support propagation of E1-deleted recombinant adenovirus, such as IG.Ad.MLPI.TK. Such cell lines will be used for the generation and (large-scale) production production of E1-deleted recombinant adenoviruses. Such cells, infected with recombinant adenovirus are also intended to be used *in vivo* as a local producer of recombinant adenovirus, e.g. for the treatment of solid tumors.

In addition, diploid cells of other species that are permissive for human adenovirus, such as the cotton rat (*Sigmodon hispidus*) (Pacini *et al.*, 1984), Syrian hamster (Morin *et al.*, 1987) or chimpanzee (Levrero *et al.*, 1991), will be immortalized with these constructs. Such cells, infected with recombinant adenovirus, are also intended to be used *in vivo* , for the local production of recombinant adenovirus, e.g. for the treatment of solid tumors.

Established cells.

The constructs, in particular pIG.E1A.NEO, are used to transfect established cells, e.g. A549 (human bronchial carcinoma), KB (oral carcinoma), MRC-5 (human diploid lung cell line) or GLC cell lines (small cell lung cancer) (de Leij *et al.*, 1985; Postmus *et al.*, 1988) and selected for NEO resistance. Individual colonies of resistant cells are

isolated and tested for their capacity to support propagation of E1-deleted recombinant adenovirus, such as IG.Ad.MLPI.TK. These cells are used for the generation and production of E1-deleted recombinant adenovirus. They are also used for the propagation of E1A deleted/E1B retained recombinant adenovirus.

Established cells are also co-transfected with pIG.E1A.E1B and pIG.NEO (or another NEO containing expression vector). Clones resistant to G418 are tested for their ability to support propagation of E1 deleted recombinant adenovirus, such as IG.Ad.MLPI.TK and used for the generation and production of E1 deleted recombiant adenovirus and will be applied *in vivo* for local production of recombinant virus, as described for the diploid cells (see above).

All cell lines, including transformed diploid cell lines or NEO-resistant established lines, can be used as the basis for the generation of 'next generation' packaging cells lines, that support propagation of E1-defective recombinant adenoviruses, that also carry deletions in other genes, such as E2A and E4. Moreover, they will provide the basis for the generation of minimal adenovirus vectors as disclosed herein.

E2 expressing cell lines

Packaging cells expressing E2A sequences are and will be used for the generation and (large scale) production of E2A-deleted recombinant adenovirus.

The newly generated human adenovirus packaging cell lines or cell lines derived from species permissive for human adenovirus (E2A or ts125E2A; E1A + E2A; E1A + E1B + E2A; E1A + E2A/ts125; E1A + E1B + E2A/ts125) or non-permissive cell lines such as monkey cells (hrE2A or hr + ts125E2A; E1A + hrE2A; E1A + E1B + hrE2A; E1A + hrE2A/ts125; E1A + E1B + hrE2A/ts125) are and will be used for the generation and (large scale) production of E2A deleted recombinant adenovirus vectors. In addition, they will be applied *in vivo* for local production of

recombinant virus, as described for the diploid cells (see above).

Use of intracellulair E2 expressing vectors.

Minimal adenovirus vectors are generated using the helper functions provided in trans by packaging-deficient replicating helper molecules. The adenovirus-derived ITR sequences serve as origins of DNA replication in the presence of at least the E2-gene products. When the E2 gene products are expressed from genes in the vector genome (N.B. the gen(s) must be driven by an E1-independent promoter), the vector genome can replicate in the target cells. This will allow an significantly increased number of template molecules in the target cells, and, as a result an increased expression of the genes of interest encoded by the vector. This is of particular interest for approaches of gene therapy in cancer.

Applications of intracellular amplification of linear DNA fragments.

A similar approach could also be taken if amplification of linear DNA fragments is desired. DNA fragments of known or unknown sequence could be amplified in cells containing the E2-gene products if at least one ITR sequence is located near or at its terminus. There are no apparent constraints on the size of the fragment. Even fragments much larger than the adenovirus genome (36 kb) should be amplified using this approach. It is thus possible to clone large fragments in mammalian cells without either shuttling the fragment into bacteria (such as E.coli) or use the polymerase chain reaction (P.C.R.). At the end stage of an productive adenovirus infection a single cell can contain over 100,000 copies of the viral genome. In the optimal situation, the linear DNA fragments can be amplified to similar levels. Thus, one should be able to extract more than 5 µg of DNA fragment per 10 million cells (for a 35-kbp fragment). This system can be used to express heterologous proteins (equivalent to the Simian

Virus 40-based COS-cell system) for research or for therapeutic purposes. In addition, the system can be used to identify genes in large fragments of DNA. Random DNA fragments may be amplified (after addition of ITRs) and expressed during intracellular amplification. Election or selection of those cells with the desired phenotype can be used to enrich the fragment of interest and to isolate the gene.

EXPERIMENTAL

Generation of cell lines able to *trans*complement E1 defective recombinant adenovirus vectors.

1. 911 cell line

We have generated a cell line that harbors E1 sequences of adenovirus type 5, able to trans-complement E1 deleted recombinant adenovirus.

This cell line was obtained by transfection of human diploid human embryonic retinoblasts (HER) with pAd5XhoIC, that contains nt. 80 - 5788 of Ad5; one of the resulting transformants was designated 911. This cell line has been shown to be very useful in the propagation of E1 defective recombinant adenovirus. It was found to be superior to the 293 cells. Unlike 293 cells, 911 cells lack a fully transformed phenotype, which most likely is the cause of performing better as adenovirus packaging line:

. plaque assays can be performed faster (4 - 5 days instead of 8-14 days on 293)

. monolayers of 911 cells survive better under agar overlay as required for plaque assays

. higher amplification of E1-deleted vectors

In addition, unlike 293 cells that were transfected with sheared adenoviral DNA, 911 cells were transfected using a defined construct. Transfection efficiencies of 911 cells are comparable to those of 293.

New packaging constructs.

Source of adenovirus sequences.

Adenovirus sequences are derived either from pAd5.SalB, containing nt. 80 - 9460 of human adenovirus type 5 (Bernards *et al.*, 1983) or from wild-type Ad5 DNA.

pAd5.SalB was digested with SalI and XhoI and the large fragment was religated and this new clone was named pAd5.X/S.

The pTN construct (constructed by Dr. R. Vogels, IntroGene, The Netherlands) was used as a source for the human PGK promoter and the NEO gene.

Human PGK promoter and NEO^R gene.

Transcription of E1A sequences in the new packaging constructs is driven by the human PGK promoter (Michelson *et al.*, 1983; Singer-Sam *et al.*, 1984), derived from plasmid pTN (gift of R. Vogels), which uses pUC119 (Vieira and Messing, 1987) as a backbone. This plasmid was also used as a source for NEO gene fused to the Hepatitis B Virus (HBV) poly-adenylation signal.

Fusion of PGK promoter to E1 genes (Fig. 1)

In order to replace the 5' sequences of Ad5 (ITR, origin of replication and packaging signal) by heterologous sequences we have amplified E1 sequences (nt.459 to nt. 960) of Ad5 by PCR, using primers Ea1 and Ea2 (see Table I). The resulting PCR product was digested with ClaI and ligated into pBluescript (Stratagene), predigested with ClaI and EcoRV, resulting in construct pBS.PCRI.

Vector pTN was digested with restriction enzymes EcoRI (partially) and ScaI, and the DNA fragment containing the PGK promoter sequences was ligated into pBS.PCRI digested with ScaI and EcoRI. The resulting construct pBS.PGK.PCRI contains the human PGK promoter operatively linked to Ad5 E1 sequences from nt.459 to nt. 916.

Construction of pE1A.E1B.X (Fig. 2)

pE1A.E1B.X was made by replacing the ScaI-BspEI fragment of pAT-X/S by the corresponding fragment from pBS.PGK.PCRI (containing the PGK promoter linked to E1A sequences).

pE1A.E1B.X contains the E1A and E1B coding sequences under the direction of the PGK promoter.

As Ad5 sequences from nt.459 to nt. 5788 are present in this construct, also pIX protein of adenovirus is encoded by this plasmid.

Construction of pIG.E1A.NEO (Fig. 3)

In order to introduce the complete E1B promoter and to fuse this promoter in such a way that the AUG codon of E1B 21 kD exactly functions as the AUG codon of NEO^R, we amplified the E1B promoter using primers Ea3 and Ep2, where primer Ep2 introduces an NcoI site in the PCR fragment. The resulting PCR fragment, named PCRII, was digested with HpaI and NcoI and ligated into pAT-X/S, which was predigested with HpaI and with NcoI. The resulting plasmid was designated pAT-X/S-PCR2. The NcoI - StuI fragment of pTN, containing the NEO gene and part of the Hepatitis B Virus (HBV) polyadenylation signal, was cloned into pAT-X/S-PCR2 (digested with NcoI and NruI). The resulting construct: pAT-PCR2-NEO. The poly-adenylation signal was completed by replacing the ScaI-SalI fragment of pAT-PCR2-NEO by the corresponding fragment of pTN (resulting in pAT.PCR2.NEO.p(A)). The ScaI - XbaI of pAT.PCR2.NEO.p(A) was replaced by the corresponding fragment of pIG.E1A.E1B-X, containing the PGK promoter linked to E1A genes.

The resulting construct was named pIG.E1A.NEO, and thus contains Ad5 E1 sequences (nt.459 to nt 1713) under the control of the human PGK promoter.

Construction of pIG.E1A.E1B (Fig. 4)

pIg.E1A.E1B was made by amplifying the sequences encoding the N-terminal amino acids of E1B 55kd using primers Eb1 and Eb2 (introduces a XhoI site). The resulting PCR fragment was

digested with BglII and cloned into BglII/NruI of pAT-X/S, thereby obtaining pAT-PCR3.

pIG.E1A.E1B was constructed by introducing the HBV poly(A) sequences of pIG.E1A.NEO downstream of E1B sequences of pAT-PCR3 by exchange of XbaI - SalI fragment of pIg.E1A.NEO and the XbaI XhoI fragment of pAT.PCR3.

pIG.E1A.E1B contains nt. 459 to nt. 3510 of Ad5, that encode the E1A and E1B proteins. The E1B sequences are terminated at the splice acceptor at nt.3511. No pIX sequences are present in this construct.

Construction of pIG.NEO (Fig. 5)

pIG.NEO was generated by cloning the HpaI - ScaI fragment of pIG.E1A.NEO, containing the NEO gene under the control of the Ad.5 E1B promoter, into pBS digested with EcoRV and ScaI.

This construct is of use when established cells are transfected with E1A.E1B constructs and NEO selection is required. Because NEO expression is directed by the E1B promoter, NEO resistant cells are expected to co-express E1A, which also is advantageous for maintaining high levels of expression of E1A during long-term culture of the cells.

Generation of cell lines with new packaging constructs.

Testing of constructs.

The integrity of the constructs pIG.E1A.NEO, pIG.E1A.E1B.X and pIG.Ea.E1B was assessed by restriction enzyme mapping; furthermore, parts of the constructs that were obtained by PCR analysis were confirmed by sequence analysis. No changes in the nucleotide sequence were found.

The constructs were transfected into primary BRK (Baby Rat Kidney) cells and tested for their ability to immortalize (pIG.E1A.NEO) or fully transform (pIG.E1A.E1B.X and pIG.Ea.E1B) these cells.

An overview of the generated adenovirus packaging constructs, and their ability to transform primary rodent

kidney cells (BRK), is presented in Fig. 6. The results indicate that the constructs pIG.E1A.E1B and pIG.E1A.E1B.X are able to transform BRK cells in a dose-dependent manner. The efficiency of transformation is similar for both constructs and is comparable to what was found with the construct that was used to make 911 cells, namely pAd5.XhoIC.

As expected, pIG.E1A.NEO was hardly able to immortalize BRK. However, co-transfection of an E1B expression construct (SV40.E1B) did result in a significant increase of the number of transformants (18 versus 1), indicating that E1A encoded by pIG.E1A.NEO is functional.

We conclude therefore, that the newly generated packaging constructs are suited for the generation of new adenovirus packaging lines.

Generation of new adenovirus vectors (Fig. 7).

The currently used recombinant adenovirus vectors (see patent application on E3) are deleted for E1 sequences from 459 to nt. 3328.

As construct pE1A.E1B contains Ad5 sequences 459 to nt. 3510 there is a sequence overlap of 183 nt. between E1B sequences in the packaging construct pIG.E1A.E1B and the recombinant adenovirus, e.g. pMLP.TK. The overlapping sequences were deleted from the new adenovirus vectors. In addition, non-coding sequences derived from lacZ, that are present in the original contructs, were deleted as well. This was achieved (see Fig. 6) by PCR amplification of the SV40 poly(A) sequences from pMLP.TK using primers SV40-1 (introduces a BamHI site) and SV40-2 (introduces a BglII site). In addition, Ad5 sequences present in this construct were amplified from nt 2496 (Ad5, introduces a BglII site) to nt. 2779 (Ad5-2). Both PCR fragments were digested with BglII and were ligated. The ligation product was PCR amplified using primers SV40-1 and Ad5-2. The PCR product obtained was cut with BamHI and AflII and was ligated into pMLP.TK predigested with the same enzymes. The resulting construct, named

pMLPI.TK, contains a deletion in adenovirus E1 sequences from nt 459 to nt. 3510.

The combination of the new pacakaging construct pIG.E1A.E1B and the recombinant adenovirus pMLPI.TK, which do not have any sequence overlap, are presented in Fig. 8. In this figure, also the original situation is presented, where the sequence overlap is indicated.

The absence of overlapping sequences between pIG.E1A.E1B and pMLPI.TK (Fig. 8a) excludes the possibility of homologous recombination between packaging construct and recombinant virus, and is therefore a significant improvement for production of recombinant adenovirus as compared to the original situation.

In Fig. 8b the situation is depicted for pIG.E1A.NEO and pMLPI.TK. pIG.E1A.NEO when transfected into established cells, is expected to be sufficient to support propagation of E1-deleted recombinant adenovirus. This combination does not have any sequence overlap, preventing generation of RCA by homologous recombination. In addition, this convenient packaging system allows the propagation of recombinant adenoviruses that are deleted just for E1A sequences and not for E1B sequences. Recombinant adenoviruses expressing E1B in the absence of E1A are attractive, as the E1B protein, in particular E1B 19kD, is able to prevent infected human cells from lysis by Tumor Necrosis Factor (TNF) (Gooding *et al.*, 1991).

Generation of recombinant adenovirus derived from pMLPI.TK.

Recombinant adenovirus was generated by co-transfection of 293 cells with SalI linearized pMLPI.TK DNA and ClaI linearized Ad5 wt DNA. The procedure is schematically represented in Fig. 9.

Outline of the strategy to generate packaging systems for minimal adenovirus vector

Name convention of the plasmids used:

p plasmid
I ITR (Adenovirus Inverted Terminal Repeat)
C Cytomegalovirus (CMV) Enhancer/Promoter Combination
L Firefly Luciferase Coding Sequence
hac,haw Potential hairpin that can be formed after digestion with restriction endonuclease Asp718 in its correct and in the reverse orientation, respectively (Fig. 12).

Eg. pICLhaw is a plasmid that contains the adenovirus ITR followed by the CMV-driven luciferase gene and the Asp718 hairpin in the reverse (non-functional) orientation.

1.1. Demonstration of the competence of a synthetic DNA sequence, that is capable of forming a hairpin structure, to serve as a primer for reverse strand synthesis for the generation of double-stranded DNA molecules in cells that contain and express adenovirus genes.

Plasmids pICLhac, pICLhaw, pICLI and pICL were generated using standard techniques. The schematic representation of these plasmids is shown in Figs. 13-16.

Plasmid pICL is derived from the following plasmids:

nt.1 - 457 pMLP10 (Levrero *et al.*, 1991)
nt.458 - 1218 pCMVß (Clontech, EMBL Bank no. U02451)
nt.1219 - 3016 pMLP.luc (Introgene, unpublished)
nt.3017 - 5620 pBLCAT5 (Stein and Whelan, 1989)

The plasmid has been constructed as follows:

The *tet* gene of plasmid pMLP10 has been inactivated by deletion of the BamHI-SalI fragment, to generate pMLP10ΔSB. Using primer set PCR/MLP1 and PCR/MLP3 a 210 bp fragment containing the Ad5-ITR, flanked by a synthetic SalI restriction site was amplified using pMLP10 DNA as the template. The PCR product was digested with the enzymes EcoRI and SgrAI to generate a 196 bp. fragment. Plasmid pMLP10ΔSB was digested with EcoRI and SgrAI to remove the ITR. This fragment was replaced by the EcoRI-SgrAI-treated PCR fragment to generate pMLP/SAL.

Plasmid pCMV-Luc was digested with PvuII to completion and recirculated to remove the SV40-derived poly-adenylation signal and Ad5 sequences with exception of the Ad5 left-terminus. In the resulting plasmid, pCMV-lucΔAd, the Ad5 ITR was replaced by the Sal-site-flanked ITR from plasmid pMLP/SAL by exchanging the χmnI-SacII fragments. The resulting plasmid, pCMV-lucΔAd/SAL, the Ad5 left terminus and the CMV-driven luciferase gene were isolated as an SalI-SmaI fragment and inserted in the SalI and HpaI digested plasmid pBLCAT5, to form plasmid pICL. Plasmid pICL is represented in Fig 16; its sequence is presented in Fig. 17.

Plasmid pICL contains the following features:

nt. 1-457 Ad5 left terminus (Sequence 1-457 of human adenovirus type 5)
nt. 458-969 Human cytomegalovirus enhancer and immediate early promoter (Boshart *et al.*, 1985)(from plasmid pCMVß, Clontech, Palo Alto, USA)
nt. 970-1204 SV40 19S exon and truncated 16/19S intron (from plasmid pCMVß)
nt. 1218-2987 Firefly luciferase gene (from pMLP.luc)
nt. 3018-3131 SV40 tandem poly-adenylation signals from late transcript, derived from plasmid pBLCAT5)
nt. 3132-5620 pUC12 backbone (derived from plasmid pBLCAT5)

nt. 4337-5191 ß-lactamase gene (Amp-resistence gene, reverse orientation)

Plasmid pICLhac and pIChaw

Plasmids PICLhac and pICLhaw were derived from plasmid pICL by digestion of the latter plasmid with the restriction enzyme Asp718. The linearized plasmid was treated with Calf-Intestine Alkaline Phosphatase to remove the 5' phoshate groups. The partially complementary synthetic single-stranded oligonucleotide Hp/asp1 en Hp/asp2 were annealed and phosphorylated on their 5'ends using T4-polynucleotide kinase.

The phosporylated double-stranded oligomers were mixed with the dephosporylated pICL fragment and ligated. Clones containing a single copy of the synthetic oligonucleotide inserted into the plasmid were isolated and characterized using restriction enzyme digests. Insertion of the oligonucleotide into the Asp718 site will at one junction recreate an Asp718 recognition site, whereas at the other junction the recognitionsite will be disrupted. The orientation and the integrity of the inserted oligonucleotide was verified in selected clones by sequence analyses. A clone containing the oligonucleotide in the correct orientation (the Asp718 site close tc the 3205 EcoRI site) was denoted pICLhac. A clone with the oligonucleotide in the reverse orientation (the Asp718 site close to the SV40 derived poly signal) was designated pIChaw. Plasmids pIChac and pIChaw are represented in Figs 13 and 14.

Plasmid pICLI was created from plasmid pICL by insertion of the SalI-SgrAI fragment from pICL, containing the Ad5-ITR into the Asp718 site of pICL. The 194 bp SalI-SgrAI fragment was isolated from pICL,and the cohesive ends were converted to blunt ends using E.coli DNA polymerase I (Klenow fragment) and dNTP's. The Asp718 cohesive ends were converted to blunt ends by treatment with mungbean nuclease. By ligation clones were generated thet contain the ITR in the Asp718 site of plasmid

pICL. A clone that contained the ITR fragment in the correct orientation was disignated pICLI (Fig. 15).

Generation of adenovirus Ad-CMV-hcTK. Recombinant adenovirus was constructed according to the method described in Patent application 94202322.7. Two components are required to generate a recombinant adenovirus. First, an adaptor-plasmid containing the left terminus of the adenovirus genome containing the ITR and the packaging signal, an expression cassette with the gene of interest, and a portion of the adenovirus genome which can be used for homologous recombination. In addition, adenovirus DNA is needed for recombination with the aforementioned adaptor plasmid. In the case of Ad-CMV-hcTK, the plasmid pCMV.TK was used as a basis. This plasmid contains nt. 1-455 of the adenovirus type 5 genome, nt. 456-1204 derived from pCMVß (Clontech, the PstI-StuI fragment that contains the CMV enhancer promoter and the 16S/19S intron from Simian Virus 40), the Herpes Simplex Virus thymidine kinase gene (decribed in Patent application 94202322.7), the SV40-derived polyadenylation signal (nt. 2533-2668 of the SV40 sequence), followed by the BglII-ScaI fragment of Ad5 (nt. 3328-6092 of the Ad5 sequence). These fragments are present in a pMLP10-derived (Levrero *et al.*, 1991) backbone. To generate plasmid pAD-CMVhc-TK, plasmid pCMV.TK was digested with ClaI (the unique ClaI-site is located just upstream of the TK open readingframe) and dephosphorylated with Calf-Intestine Alkaline Phospatase. To generate a hairpin structure, the synthetic oligonucleotides HP/cla2 and HP/cla2 were annealed and phopsphorylated on their 5'-OH groups with T4-polynucleotide kinase and ATP. The double-stranded ologonucleotide was ligated with the linearized vector fragment and used to transform E.coli strain "Sure". Insetion of the oligonucleotide into the ClaI site will disrupt the ClaI recognition sites. In the oligonucleotide contains a new ClaI site near one of its termini. In selected clones, the orientation and the inegrity of the inserted oligonucleotide was verified by sequence analyses. A clone containing the oligonucleotide in the

correct orientation (the ClaI site at the ITR side) was denoted pAd-CMV-hcTK. This plasmid was cotransfected with ClaI digested wild-type Adenovirus-type5 DNA into 911 cells. A recombinant adenovirus in which the CMV-hcTK expression cassette replaces the E1 sequences was isolated and propagated using standard procedures.

To study whether the hairpin can be used as a primer for reverse strand synthesis on the displaced strand after replication had started at the ITR, the plasmid pICLhac is introduced into 911 cells (human embryonic retinoblasts transformed with the adenovirus E1 region). The plasmid pICLhaw serves as a control, which contains the oligonucleotide pair HP/asp 1 and 2 in the reverse orientation but is further completely identical to plasmid pICLhac. Also included in these studies are plasmids pICLI and pICL. In the plasmid pICLI the hairpin is replaced by an adenovirus ITR. Plasmid pICL contains neither a hairpin nor an ITR sequence. These plasmids serve as controls to determine the efficiency of replication by virtue of the terminal-hairpin structure. To provide the viral products other than the E1 proteins (these are produced by the 911 cells) required for DNA replication the cultures are infected with the virus IG.Ad.MLPI.TK after transfection. Several parameters are being studied to demonstrate proper replication of the transfected DNA molecules. First, DNA extracted from the cell cultures transfected with aforementioned plasmids and infected with IG.Ad.MLPI.TK virus is being analyzed by Southern blotting for the presence of the expected replication intermediates, as well as for the presence of the duplicated genomes. Furthermore, from the transfected and IG.Ad.MLPI.TK infected cell populations virus is isolated, that is capable to transfer and express a luciferase marker gene into luciferase negative cells.

Plasmid DNA of plasmids pICLhac, pICLhaw, pICLI and pICL have been digested with restriction endonuclease SalI and treated with mungbean nuclease to remove the 4 nucleotide single-stranded extension of the resulting DNA fragment. In

this manner a natural adenovirus 5'ITR terminus on the DNA fragment is created. Subsequently, both the pICLhac and pICLhaw plasmids were digested with restriction endonuclease Asp718 to generate the terminus capable of forming a hairpin structure. The digested plasmids are introduced into 911 cells, using the standard calcium phosphate co-precipitation technique, four dishes for each plasmid. During the transfection, for each plasmid two of the cultures are infected with the IG.Ad.MLPI.TK virus using 5 infectious IG.Ad.MLPI.TK particles per cell. At twenty-hours post-transfection and forty hours post-transfection one Ad.tk-virus-infected and one uninfected culture are used to isolate small molecular-weight DNA using the procedure devised by Hirt. Aliquots of isolated DNA are used for Southern analysis. After digestion of the samples with restriction endonuclease EcoRI using the luciferase gene as a probe a hybridizing fragment of approx. 2.6kb is detected only in the samples from the adenovirus infected cells transfected with plasmid pICLhac. The size of this fragment is consistent with the anticipated duplication of the luciferase marker gene. This supports the conclusions that the inserted hairpin is capable to serve as a primer for reverse strand synthesis. The hybridizing fragment is absent if the IG.Ad.MLPI.TK virus is omitted, or if the hairpin oligonucleotide has been inserted in the reverse orientation.

The restriction endonuclease DpnI recognizes the tetranucleotide sequence 5'-GATC-3', but cleaves only methylated DNA, (that is, only (plasmid) DNA propagated in, and derived, from E.coli, not DNA that has been replicated in mammalian cells). The restriction endonuclease MboI recognizes the same sequences, but cleaves only unmethylated DNA (viz. DNA propagated in mammalian cells). DNA samples isolated from the transfected cells are incubated with MboI and DpnI and analysed with Southern blots. These results demonstrate that only in the cells transfected with the pICLhac and the pICLI plasmids large DpnI-resistant fragments are present, that are absent in the MboI treated samples. These data demonstrate

that only after transfection of plasmids pICLI and pICLhac replication and duplication of the fragments occur.

These data demonstrate that in adenovirus-infected cells linear DNA fragments that have on one terminus an adenovirus-derived inverted terminal repeat (ITR) and at the other terminus a nucleotide sequence that can anneal to sequences on the same strand, when present in single-stranded form thereby generate a hairpin structure, and will be converted to structures that have inverted terminal repeat sequences on both ends. The resulting DNA molecules will replicate by the same mechanism as the wild type adenovirus genomes.

1.2. Demonstration that the DNA molecules that contain a luciferase marker gene, a single copy of the ITR, the encapsidation signal and a synthetic DNA sequence, that is capable of forming a hairpin structure, are sufficient to generate DNA molecules that can be encapsidated into virions.

To demonstrate that the above DNA molecules containing two copies of the CMV-luc marker gene can be encapsidated into virions, virus is harvested from the remaining two cultures via three cycles of freeze-thaw crushing and is used to infect murine fibroblasts. Forty-eight hours after infection the infected cells are assayed for luciferase activity. To exclude the possibility that the luciferase activity has been induced by transfer of free DNA, rather than via virus particles, virus stocks are treated with DNaseI to remove DNA contaminants. Furthermore, as an additional control, aliquots of the virus stocks are incubated for 60 minutes at 56°C. The heat treatment will not affect the contaminating DNA, but will inactivate the viruses. Significant luciferase activity is only found in the cells after infection with the virus stocks derived from IG.Ad.MLPI.TK-infected cells transfected with the pICLhc and pICLI plasmids. Neither in the non-infected cells, nor in the infected cells transfected with the pICLhw and pICL significant luciferase activity can be demonstrated. Heat inactivation, but not DNaseI treatment, completely eliminates luciferase expression, demonstrating that adenovirus

particles, and not free (contaminating) DNA fragments are responsible for transfer of the luciferase reporter gene.

These results demonstrate that these small viral genomes can be encapsidated into adenovirus particles and suggest that the ITR and the encapsidation signal are sufficient for encapsidation of linear DNA fragments into adenovirus particles. These adenovirus particles can be used for efficient gene transfer. When introduced into cells that contain and express at least part of the adenovirus genes (viz. E1, E2, E4, and L, and VA), recombinant DNA molecules that consist of at least one ITR, at least part of the encapsidation signal as well as a synthetic DNA sequence, that is capable of forming a hairpin structure, have the intrinsic capacity to autonomously generate recombinant genomes which can be encapsidated into virions. Such genomes and vector system can be used for gene transfer.

1.3. Demonstration that DNA molecules which contain nucleotides 3510 - 35953 (viz. 9.7 - 100 map units) of the adenovirus type 5 genome (thus lack the E1 protein-coding regions, the right-hand ITR and the encapsidation sequences) and a terminal DNA sequence that is complementary to a portion of the same strand of the DNA molecule when present in single-stranded form other than the ITR, and as a result is capable of forming a hairpin structure, can replicate in 911 cells.

In order to develop a replicating DNA molecule that can provide the adenovirus products required to allow the above mentioned ICLhac vector genome and alike minimal adenovectors to be encapsidated into adenovirus particles by helper cells, the Ad-CMV-hcTK adenoviral vector has been developed. Between the CMV enhancer/promoter region and the thymidine kinase gene the annealed oligonucleotide pair HP/cla 1 and 2 is inserted. The vector Ad-CMV-hcTK can be propagated and produced in 911 cell using standard procedures. This vector is grown and propagated exclusively as a source of DNA used for transfection. DNA of the adenovirus Ad-CMV-hcTK is isolated from virus particles that had been purified using CsCl density-gradient centrifugation by standard techniques. The

virus DNA has been digested with restriction endonuclease ClaI. The digested DNA is size-fractionated on an 0.7% agarose gel and the large fragment is isolated and used for further experiments. Cultures of 911 cells are transfected large ClaI-fragment of the Ad-CMV-hcTK DNA using the standard calcium phosphate co-precipitation technique. Much like in the previous experiments with plasmid pICLhac, the AD-CMV-hc will replicate starting at the right-hand ITR. Once the l-strand is displaced, a hairpin can be formed at the left-hand terminus of the fragment. This facilitates the DNA polymerase to elongate the chain towards the right-hand-side. The process will proceed until the displaced strand is completely converted to its double-stranded form. Finally, the right-hand ITR will be recreated, and in this location the normal adenovirus replication-initiation and elongation will occur. Note that the polymerase will read through the hairpin, thereby duplicating the molecule. The input DNA molecule of 33250 bp, that had on one side an adenovirus ITR sequence and at the other side a DNA sequence that had the capacity to form a hairpin structure, has now been duplicated, in a way that both ends contain an ITR sequence. The resulting DNA molecule will consist of a palindromic structure of approximately 66500 bp.

This structure can be detected in low-molecular weight DNA extracted from the transfected cells using Southern analysis. The palindromic nature of the DNA fragment can be demonstrated by digestion of the low-molecular weight DNA with suitable restriction endonucleases and Southern blotting with the HSV-TK gene as the probe. This molecule can replicate itself in the transfected cells by virtue of the adenovirus gene products that are present in the cells. In part, the adenovirus genes are expressed from templates that are integrated in the genome of the target cells (viz. the E1 gene products), the other genes reside in the replicating DNA fragment itself. Note however, that this linear DNA fragment cannot be encapsidated into virions. Not only does it lack all

the DNA sequences required for encapsidation, but also is its size much too large to be encapsidated.

1.4. Demonstration that DNA molecules which contain nucleotides 3503 - 35953 (viz. 9.7 - 100 map units) of the adenovirus type 5 genome (thus lack the E1 protein-coding regions, the right-hand ITR and the encapsidation sequences) and a terminal DNA sequence that is complementary to a portion the same strand of the DNA molecule other than the ITR, and as a result is capable of forming a hairpin structure, can replicate in 911 cells and can provide the helper functions required to encapsidate the pICLI and PICLhac derived DNA fragments.

The next series of experiments aim to demonstrate that the DNA molecule described in part 1.3 could be used to encapsidate the minimal adenovectors described in part 1.1 and 1.2.

In the experiments the large fragment isolated after endonuclease ClaI-digestion of Ad-CMV-hcTK DNA is introduced into 911 cells (conform the experiments described in part 1.3) together with endonuclease SalI, mungbean nuclease, endonuclease Asp718-treated plasmid pICLhac, or as a control similarly treated plasmid pICLhaw. After 48 hours virus is isolated by freeze-thaw crushing of the transfected cell population. The virus-preparation is treated with DNaseI to remove contaminating free DNA. The virus is used subsequently to infect Rat2 fibroblasts. Forty-eight hours post infection the cells are assayed for luciferase activity. Only in the cells infected with virus isolated from the cells transfected with the pICLhac plasmid, and not with the pICLhaw plasmid, significant luciferase activity can be demonstrated. Heat-inactivation of the virus prior to infection completely abolishes the luciferase activity, indicating that the luciferase gene is transferred by a viral particle. Infection of 911 cell with the virus stock did not result in any cytopathological effects, demonstrating that the pICLhac is produced without any infectious helper virus that can be propagated on 911 cells. These results demonstrate that the

proposed method can be used to produce stocks of minimal-adenoviral vectors, that are completely devoid of infectious helper viruses that are able to replicate autonomously on adenovirus-transformed human cells or on non-adenovirus transformed human cells.

Besides the system described in this application, another approach for the generation of minimal adenovirus vectors has been disclosed in WO 94/12649. The method described in WO 94/12649 exploits the function of the protein IX for the packaging of minimal adenovirus vectors (Pseudo Adenoviral Vectors (PAV) in the terminology of WO 94/12649). PAVs are produced by cloning an expression plasmid with the gene of interest between the left-hand (including the sequences required for encapsidation) and the right-hand adenoviral ITRs. The PAV is propagated in the presence of a helper virus. Encapsidation of the PAV is preferred compared the helper virus because the helper virus is partially defective for packaging. (Either by virtue of mutations in the packaging signal or by virtue of its size (virus genomes greater than 37.5 kb package inefficiently). In addition, the authors propose that in the absence of the protein IX gene the PAV will be preferentially packaged. However, neither of these mechanisms appear to be sufficiently restrictive to allow packaging of only PAVs/minimal vectors. The mutations proposed in the packaging signal diminish packaging, but do not provide an absolute block as the same packaging-activity is required to propagate the helper virus. Also neither an increase in the size of the helper virus nor the mutation of the protein IX gene will ensure that PAV is packaged exclusively. Thus, the method described in WO 94/12649 is unlikely to be useful for the production of helper-free stocks of minimal adenovirus vectors/PAVs.

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Table I

Primers used for PCR amplification of DNA fragments used for generation of constructs described in this patent application.

Ea-1	CGTGTAGTGTATTTATACCCG	PCR amplification Ad5 nt459 ->
Ea-2	TCGTCACTGGGTGGAAAGCCA	PCR amplification Ad5 nt960 <-
Ea-3	TACCCGCCGTCCTAAAATGGC	nt1284-1304 of Ad5 genome
Ea-5	TGGACTTGAGCTGTAAACGC	nt1514-1533 of Ad5 genome
Ep-2	GCCTCCATGGAGGTCAGATGT	nt1721-1702 of Ad5; introduction of NcoI site
Eb-1	GCTTGAGCCCGAGACATGTC	nt3269-3289 of Ad5 genome
Eb-2	CCCCTCGAGCTCAATCTGTATCTT	nt3508-3496 of Ad5 genome; introduction of XhoI site
SV40-1	GGGGGATCCGAACTTGTTTATTGCAGC	Introduction BamHI site (nt2182-2199 of pMLP.TK) adaption of ecombinant adenoviruses
SV40-2	GGGAGATCTAGACATGATAAGATAC	Introduction BglII site (nt2312-2297 of pMLP.TK)
Ad5-1	GGGAGATCTGTACTGAAATGTGTGGGC	Introduction BglII site (nt2496-2514 of pMLP.TK)
Ad5-2	GGAGGCTGCAGTCTCCAACGGCGT	nt2779-2756 of pMLP.TK
ITR1	GGGGGATCCTCAAATCGTCACTTCCGT	nt35737-35757 of Ad5 (introduction of BamHI site)
ITR2	GGGGTCTAGACATCATCAATAATATAC	nt35935-35919 of Ad5 (introduction of XbaI site)

PCR primers sets to be used to create the SalI and Asp718 sites juxtaposed to the ITR sequences.

PCR/MLP1	GGCGAATTCGTCGACATCATCAATAATATACC	(Ad5 nt. 10-18)
PCR/MLP2	GGCGAATTCGGTACCATCATCAATAATATACC	(Ad5 nt. 10-18)
PCR/MLP3	CTGTGTACACCGGCGCA	(Ad5 nt.200-184)

Synthetic oligonucleotide pair used to generate a synthetic hairpin, recreates an Asp718 site at one of the termini if inserted in Asp718 site:

HP/asp1 5'-GTACACTGACCTAGTGCCGCCCGGGCAAAGCCCGGGCGGCACTAGGTCAG

HP/asp2 5'-GTACCTGACCTAGTGCCGCCCGGGCTTTGCCCGGGCGGCACTAGGTCAGT

Synthetic oligonucleotide pair used to generate a synthetic hairpin, contains the ClaI recognition site to be used for hairpin formation.

HP/cla1 5'-GTACATTGACCTAGTGCCGCCCGGGCAAAGCCCGGGCGGCACTAGGTCAATCGAT

HP/cla2 5'-GTACATCGATTGACCTAGTGCCGCCCGGGCTTTGCCCGGGCGGCACTAGGTCAAT

CLAIMS

1. A recombinant nucleic acid molecule based on or derived from an adenovirus having at least a functional encapsidating signal and at least one functional Inverted Terminal Repeat or a functional fragment or derivative thereof and having no overlapping sequences which allow for homologous recombination leading to replication competent virus in a cell into which it is transferred.

2. A recombinant nucleic acid molecule according to claim 1 being in a linear form and comprising an Inverted Terminal Repeat at or near both termini.

3. A recombinant nucleic acid molecule according to claim 1 being in a linear and essentially single stranded form and comprising at the 3' terminus a sequence complementary to an upstream part of the same strand of said nucleic acid molecule, said sequence being capable of base-pairing with said part in a way to be able to function as a start-site for a nucleic acid polymerase.

4. A recombinant nucleic acid molecule according to claim 3, comprising all adenovirus derived genetic information necessary for replication, except for a functional encapsidation signal.

5. A recombinant nucleic acid molecule derived from the nucleic acid molecule according to claim 4 resulting from the action of a nucleic acid polymerase on said nucleic acid molecule according to claim 4.

6. A recombinant nucleic acid molecule according to claim 5 having an Inverted Terminal Repeat at both termini.

7. A recombinant nucleic acid molecule according to anyone of the aforegoing claims comprising a host range mutation.

8. A recombinant nucleic acid molecule according to anyone of the aforegoing claims comprising a mutated E2 region rendering at least one of its products temperature sensitive.

9. A recombinant nucleic acid molecule according to anyone of the aforegoing claims comprising an E2 region under the control of an inducible promoter.

10. A packaging cell for packaging adenovirus derived nucleic acid molecules, which packaging cell has been provided with one or more recombinant nucleic acid molecules which provide said cell with the ability to express adenoviral gene products derived from at least the E1A region.

11. A packaging cell for packaging adenovirus derived nucleic acid molecules, which packaging cell has been provided with one or more recombinant nucleic acid molecules which provide said cell with the ability to express adenoviral gene products derived from at least both the E1A and the E2A region.

12. A packaging cell according to claim 11, wherein the recombinant nucleic acid molecule encoding the E2A region is under control of an inducible promoter.

13. A packaging cell according to claim 11 or 12, wherein the recombinant nucleic acid molecule encoding the E2A region is mutated so that at least one of its products is temperature sensitive.

14. A cell according to anyone of claims 10-13, which does not have the ability to express E1B products.

15. A cell according to claim 14, wherein the genetic information encoding E1B products is not present.

16. A cell according to claim 10, further comprising the region coding for E1B.

17. A cell according to claim 10, further comprising a marker gene.

18. A cell according to claim 17, whereby the marker gene is under control of the E1B responsive promoter.

19. A packaging cell harbouring nucleotides 80-5788 of the human Adenovirus 5 genome.

20. A packaging cell harbouring nucleotides 459-1713 of the human Adenovirus 5 genome.

21. A packaging cell harbouring nucleotides 459-3510 of the human Adenovirus 5 genome.

22. A cell according to anyone of claims 10-13, which does not have the ability to express the 21kD E1B product.

23. A cell according to claim 22, wherein the genetic information encoding the 21kD E1B product is not present.

24. A cell according to anyone of claims 10-23 which is a diploid cell.

25. A cell according to anyone of claims 10-24 which is of non-human origin.

26. A cell according to anyone of claims 10-25 which is of monkey origin.

27. A cell according to claim 19 as deposited under no 95062101 at the ECACC.

28. A recombinant nucleic acid molecule according to anyone of claims 1-9 being a DNA molecule.

29. A recombinant nucleic acid molecule based on or derived from an adenovirus, having at least a deletion of nucleotides 459-3510 of the E1 region.

30. A recombinant nucleic acid molecule based on or derived from an adenovirus, having a deletion of nucleotides 459-1713 of the E1 region.

31. An adenovirus-like particle comprising a recombinant nucleic acid molecule according to anyone of claims 1-9.

32. A cell comprising a recombinant nucleic acid molecule according to anyone of claims 1-9.

33. A recombinant nucleic acid according to claims 1-3, comprising functional E2A end E2B genes or functional fragments or derivatives thereof under control of an E1A independent promoter.

34. A cell according to claim 26 which comprises a host range mutated E2A region of an adenovirus.

35. A method for intracellular amplification comprising the steps of providing a cell with a linear DNA fragment to be amplified, which fragment is provided with at least a functional part or derivative of an Inverted Terminal Repeat at one terminus and providing said cell with functional E2 derived products necessary for replication of said fragment

and allowing said fragment to be acted upon by a DNA polymerase.

36. A method according to claim 35 whereby the cell is provided with genetic material encoding both E2A and E2B products.

37. A method according to claim 35 or 36 whereby a hairpin-like structure is provided at the terminus of the DNA fragment opposite the Inverted Terminal Repeat.

ABSTRACT

Title: Improved materials derived from recombinant adenovirus to be used in gene therapy.

The invention provides improved methods and products based on adenoviral materials which can advantageously be used in for instance gene therapy. In one aspect an adenoviral vector is provided which has no overlap with a suitable packaging cell line which is another aspect of the invention. This combination excludes the possibility of homologous recombination, thereby excluding the possibility of the frormation of replication competent adenovirus. In another aspect an adenovirus based helper construct which by its size is incapable of being encapsidated. This helper virus can be transferred into any suitable host cell making it a packaging cell. Further a number of useful mutations to adenoviral based materials and combinations of such mutations are disclosed, which all have in common the safety of the methods and the products, in particular avoiding the production of replication competent adenovirus and/or interference with the immune system. Further a method of intracellular amplification is provided.

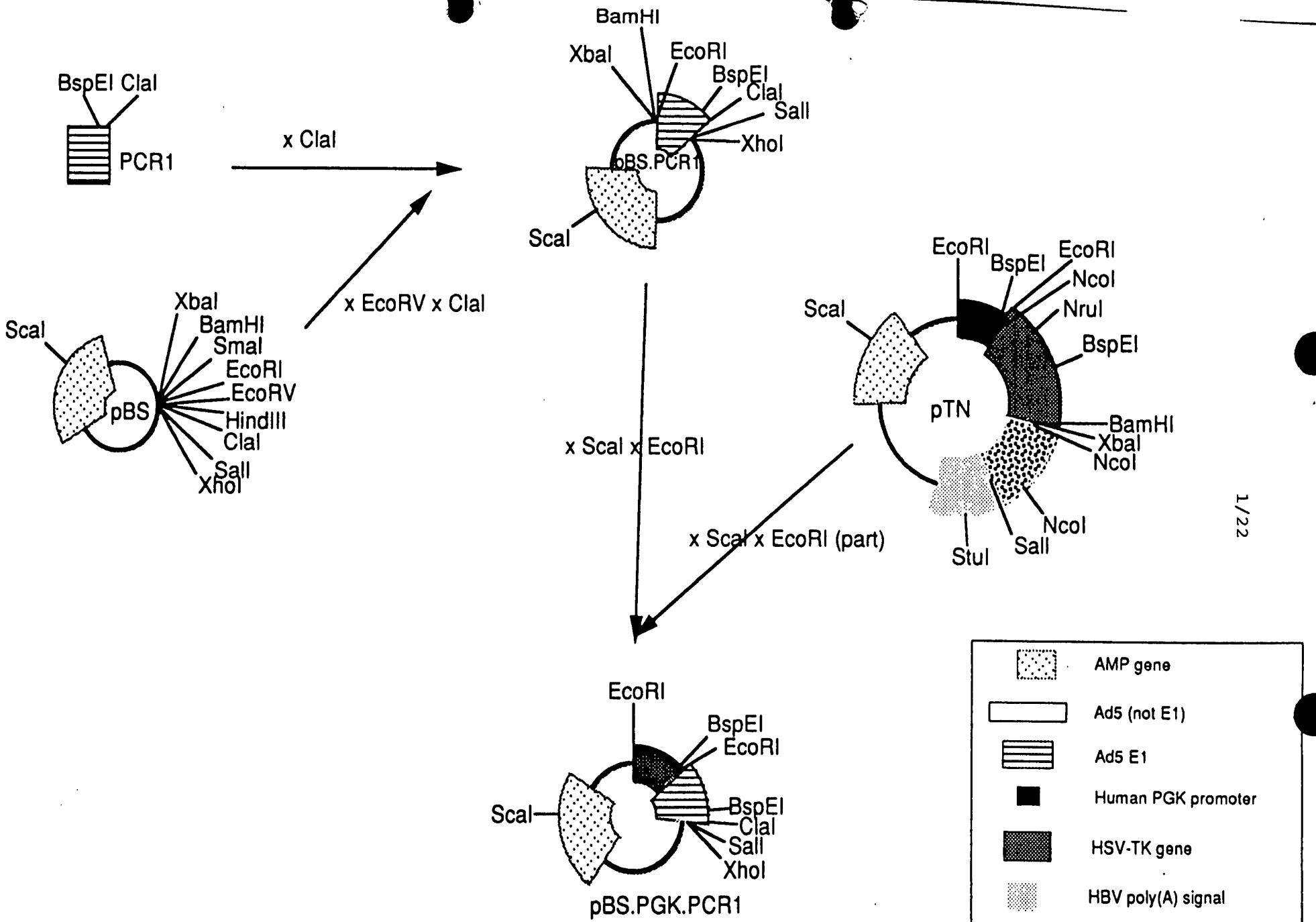

Figure 1

Construction of pBS.PGK.PCRI

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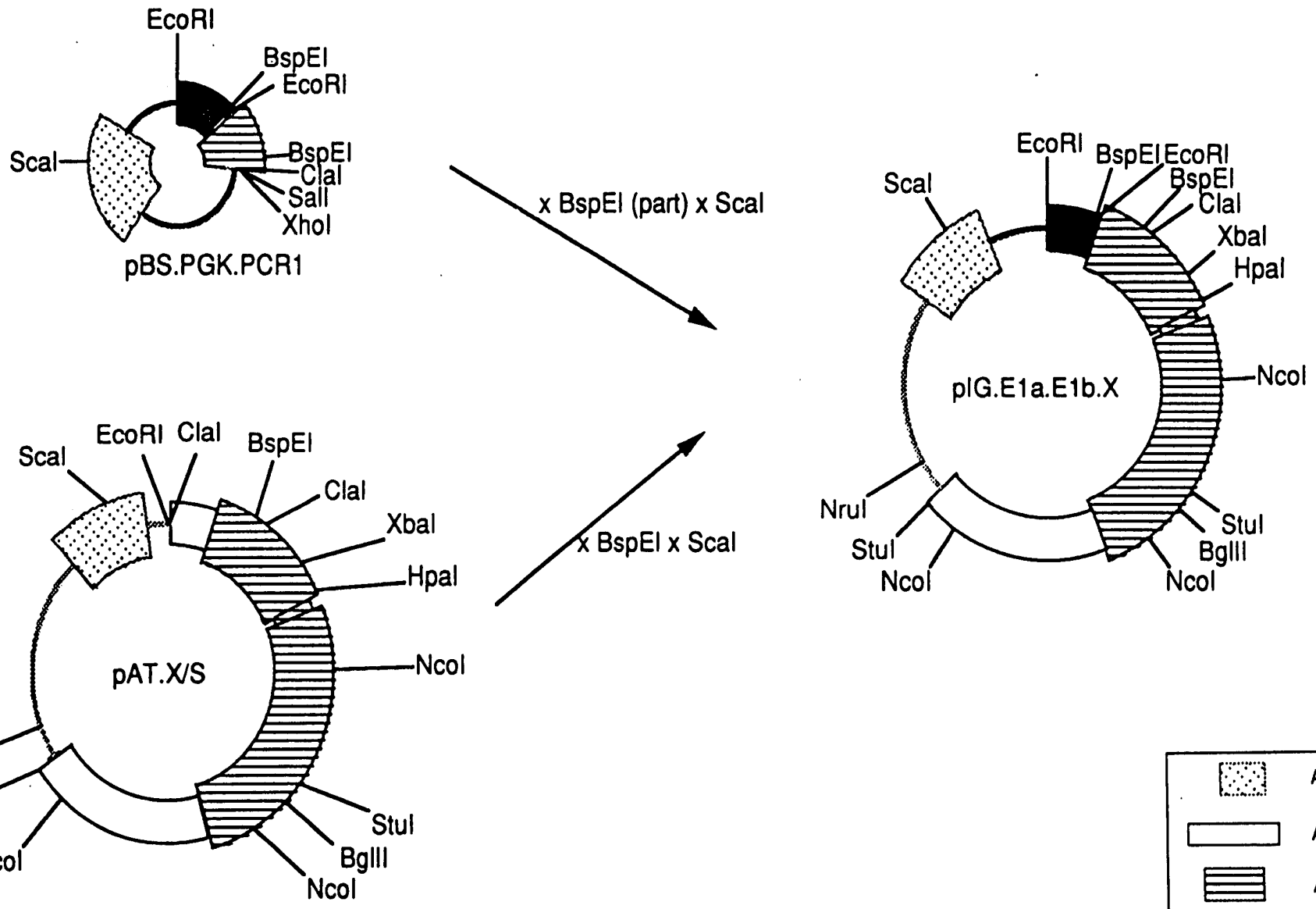

Figure 2

Construction of pE1a.E1b.X

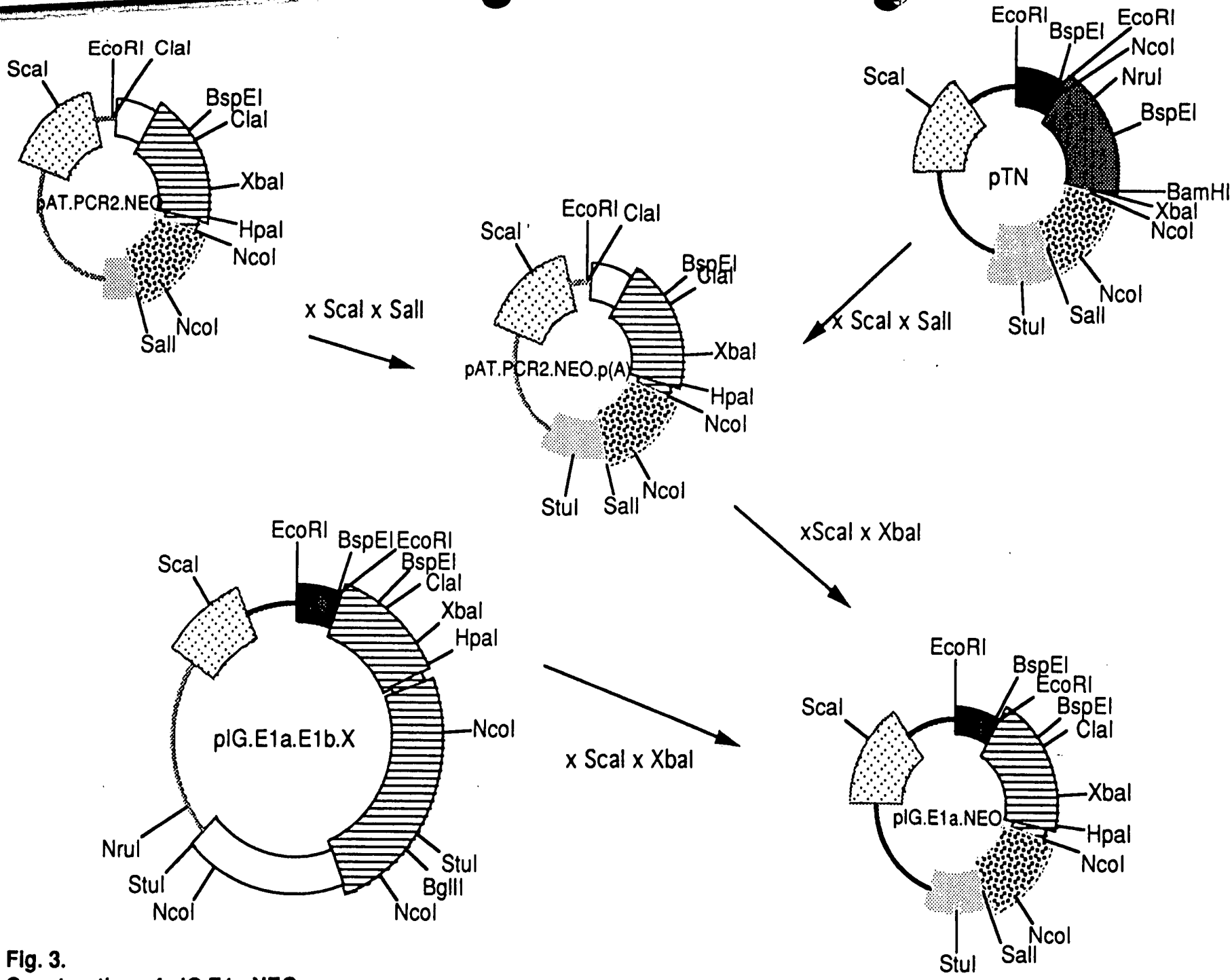

Fig. 3.
Construction of pIG.E1a.NEO

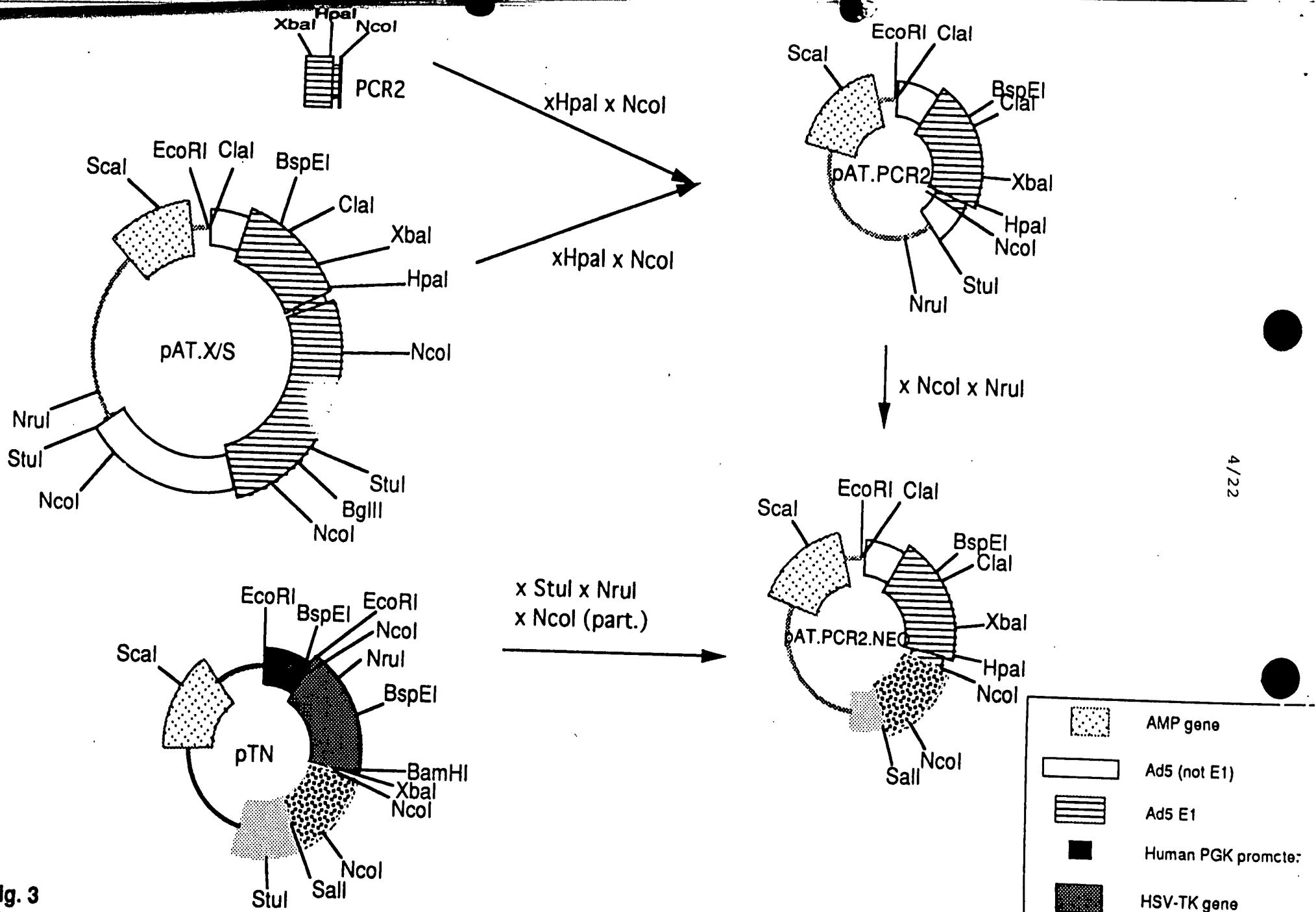

Fig. 3

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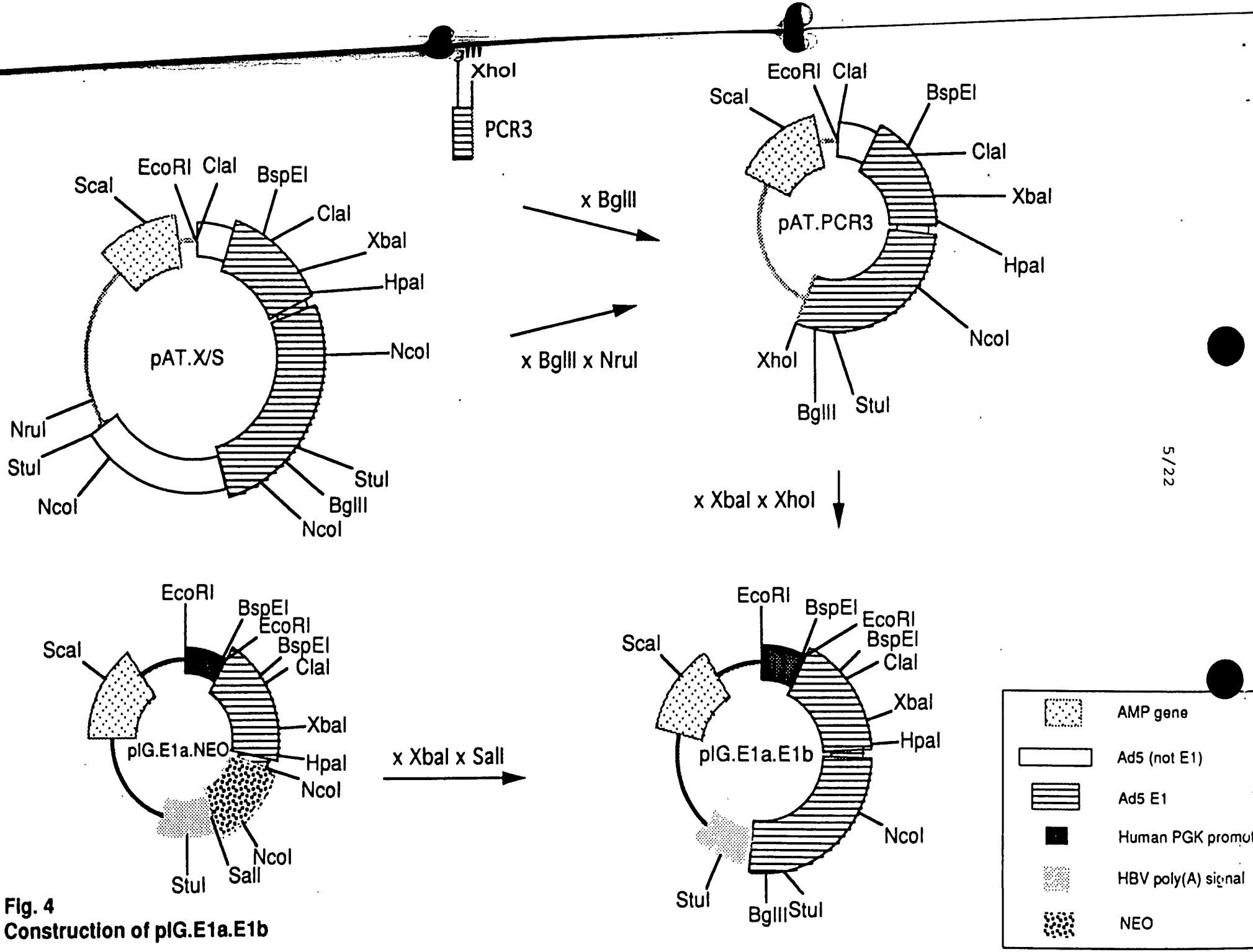
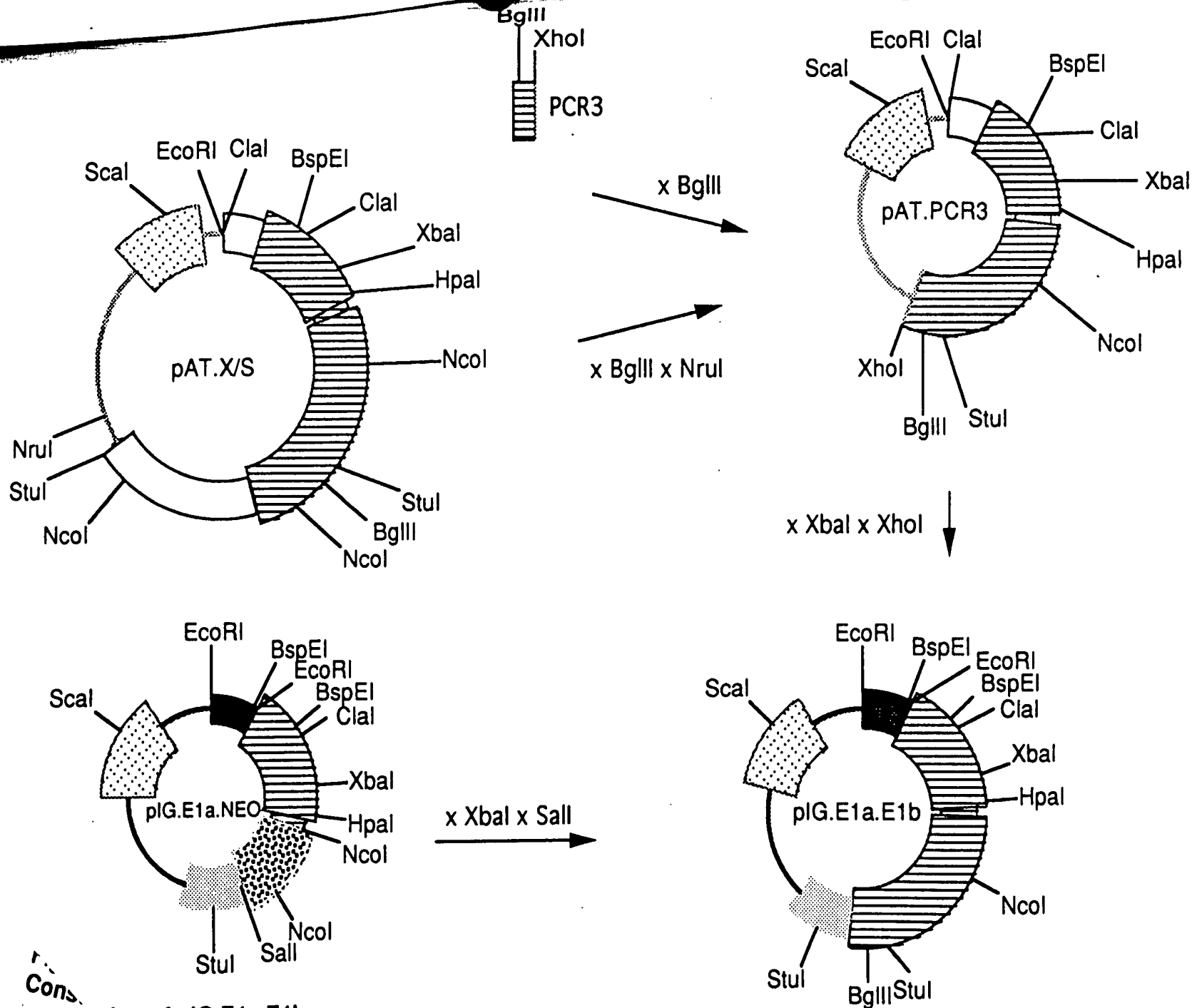


Fig. 4
Construction of pIG.E1a.E1b



Cons ction of pIG.E1a.E1b

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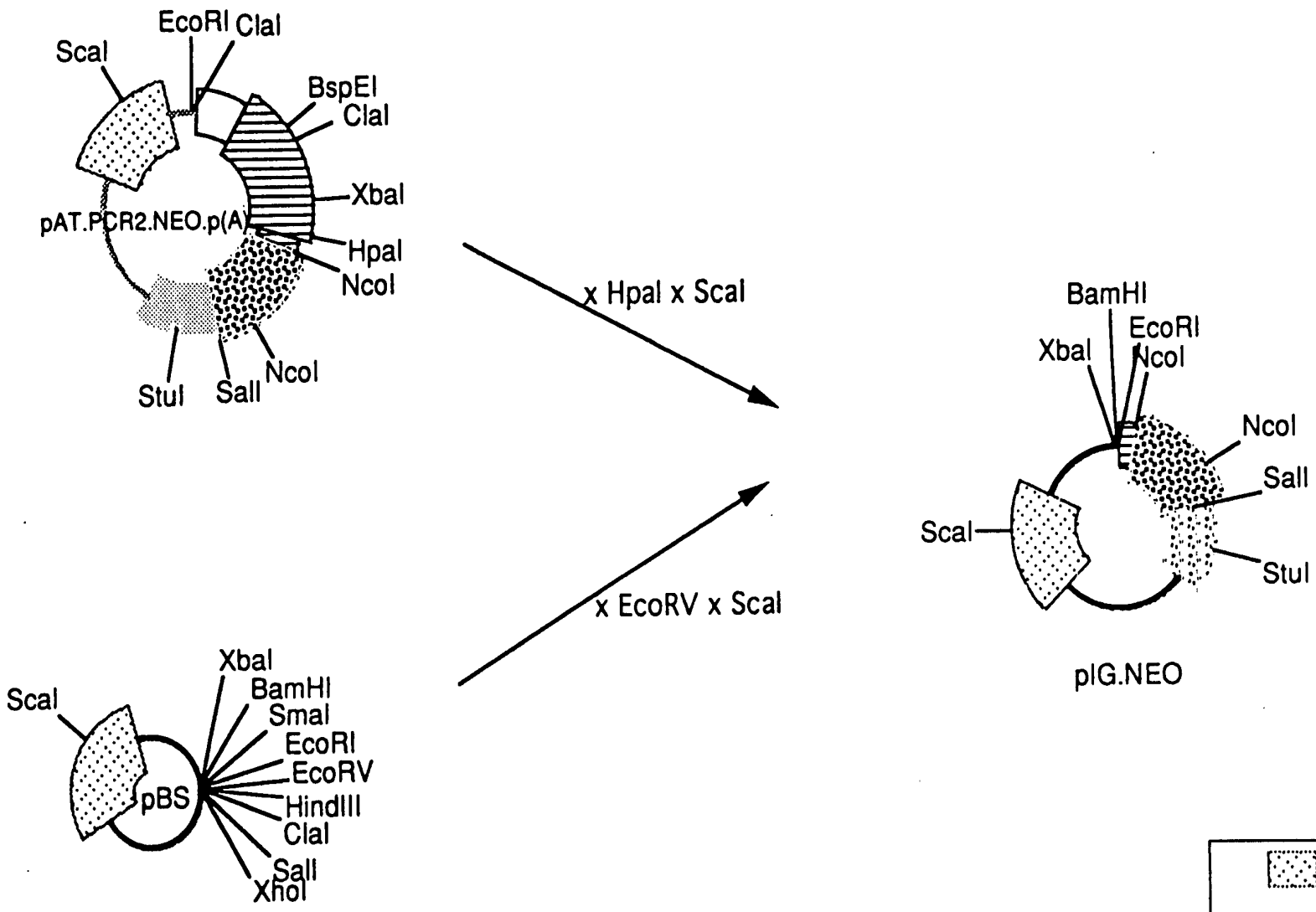

Fig. 5
Construction of pIG.NEO

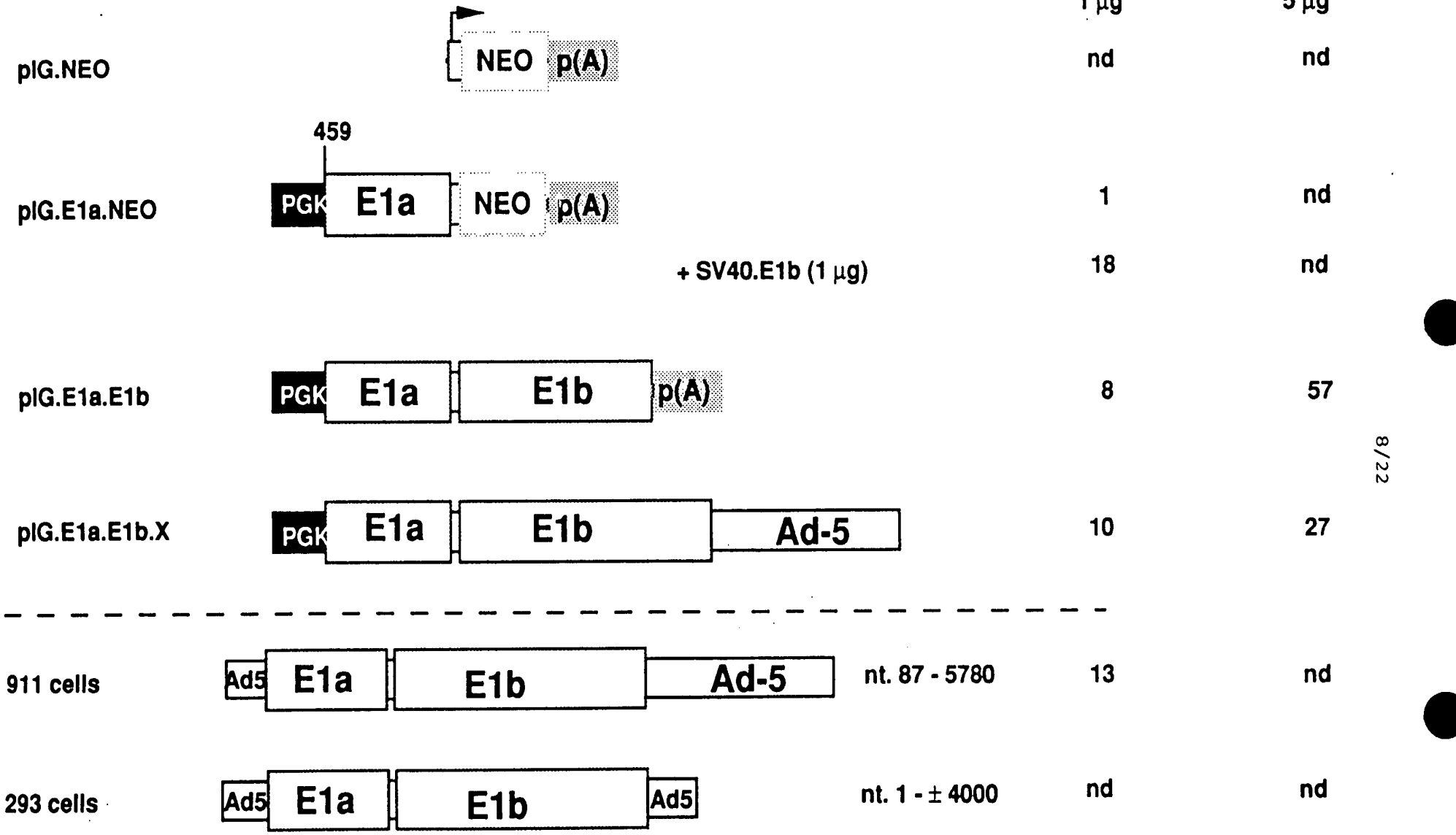

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Figure 6
Overview of available adenovirus packaging constructs and assessment of their capacity to transform primary kidney cells

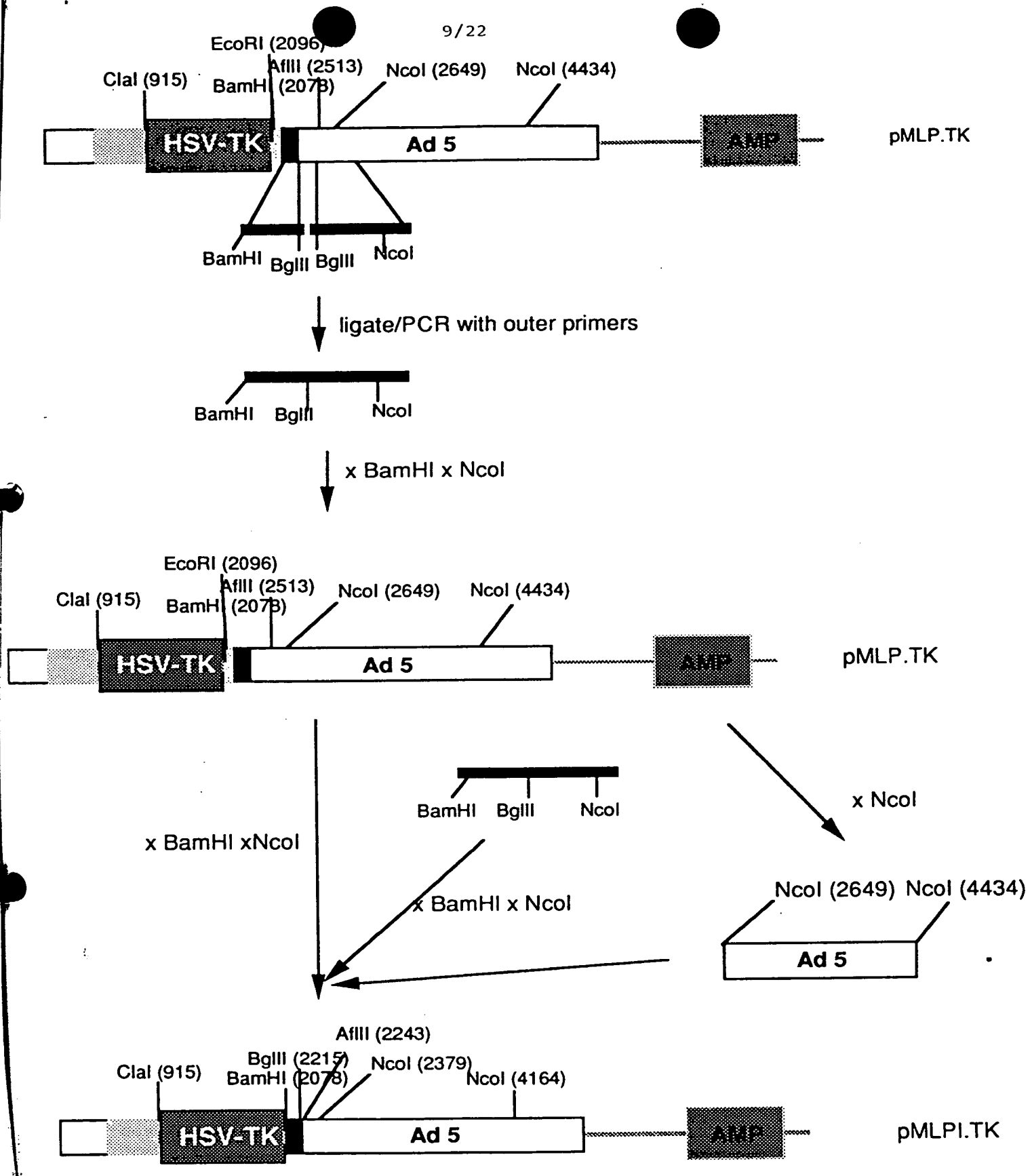


Figure 7.
Construction of pMLPI.TK from pMLP.TK

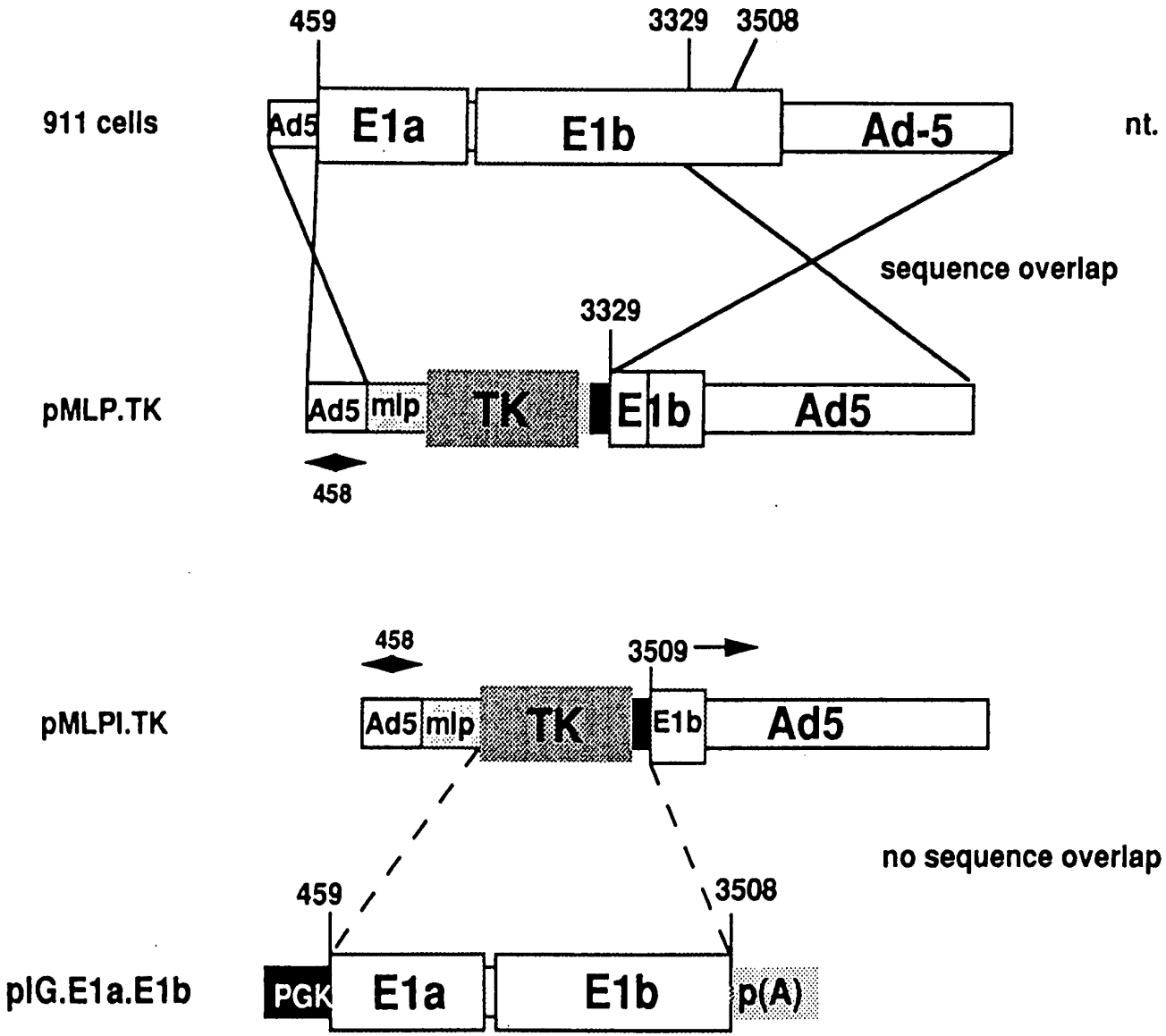


Figure 8a
Packaging system based on primary cells

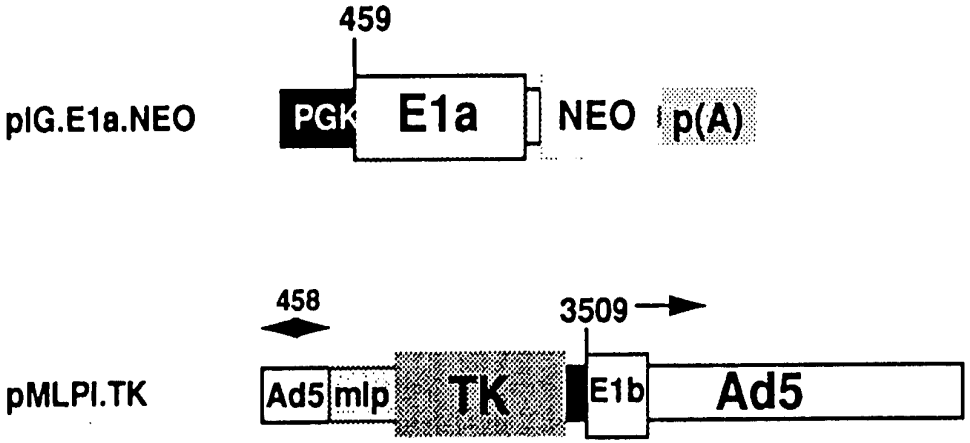


Figure 8.b
Packaging system based on established cell lines: transfection with E1a and selection with G418

Construction of recombinant adenovirus

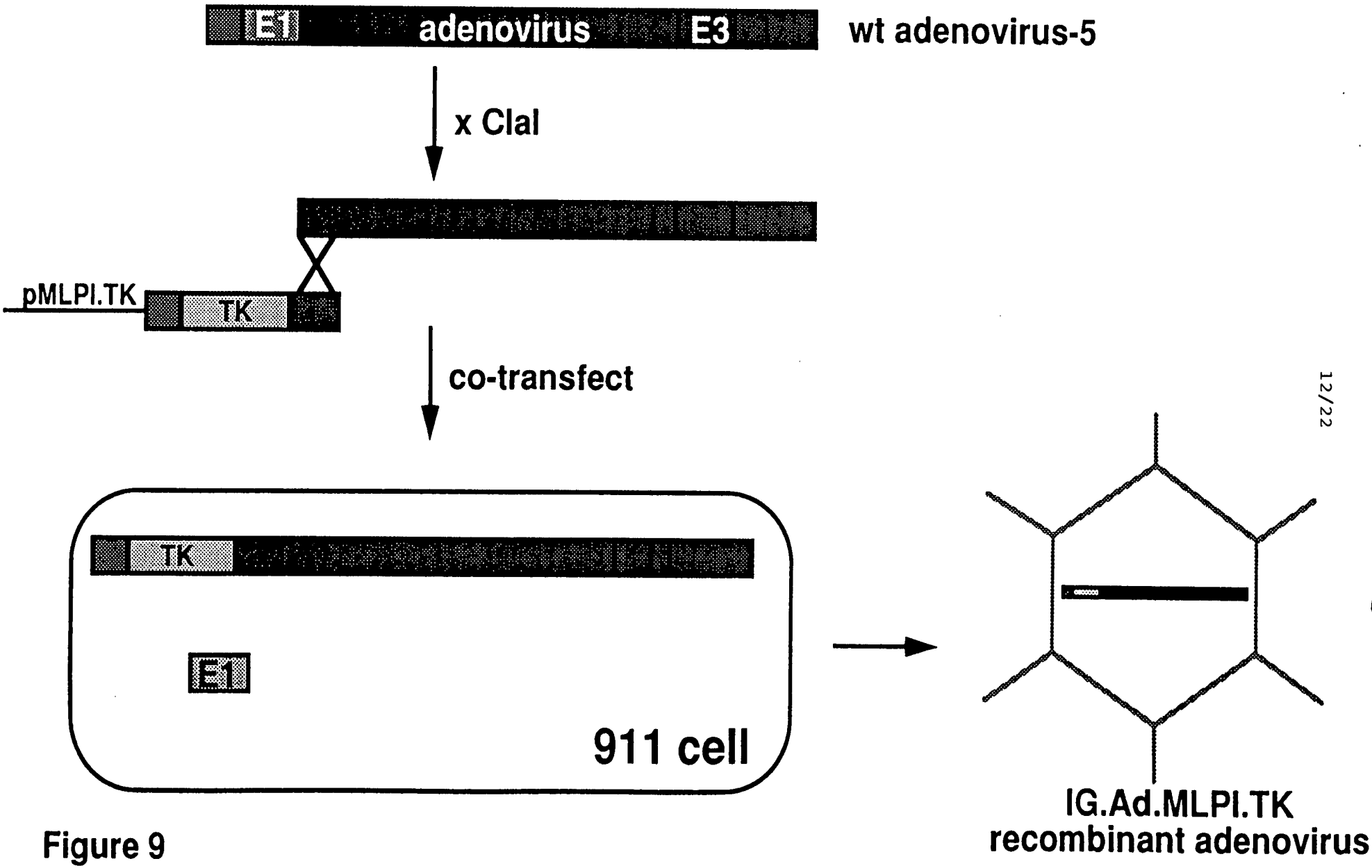
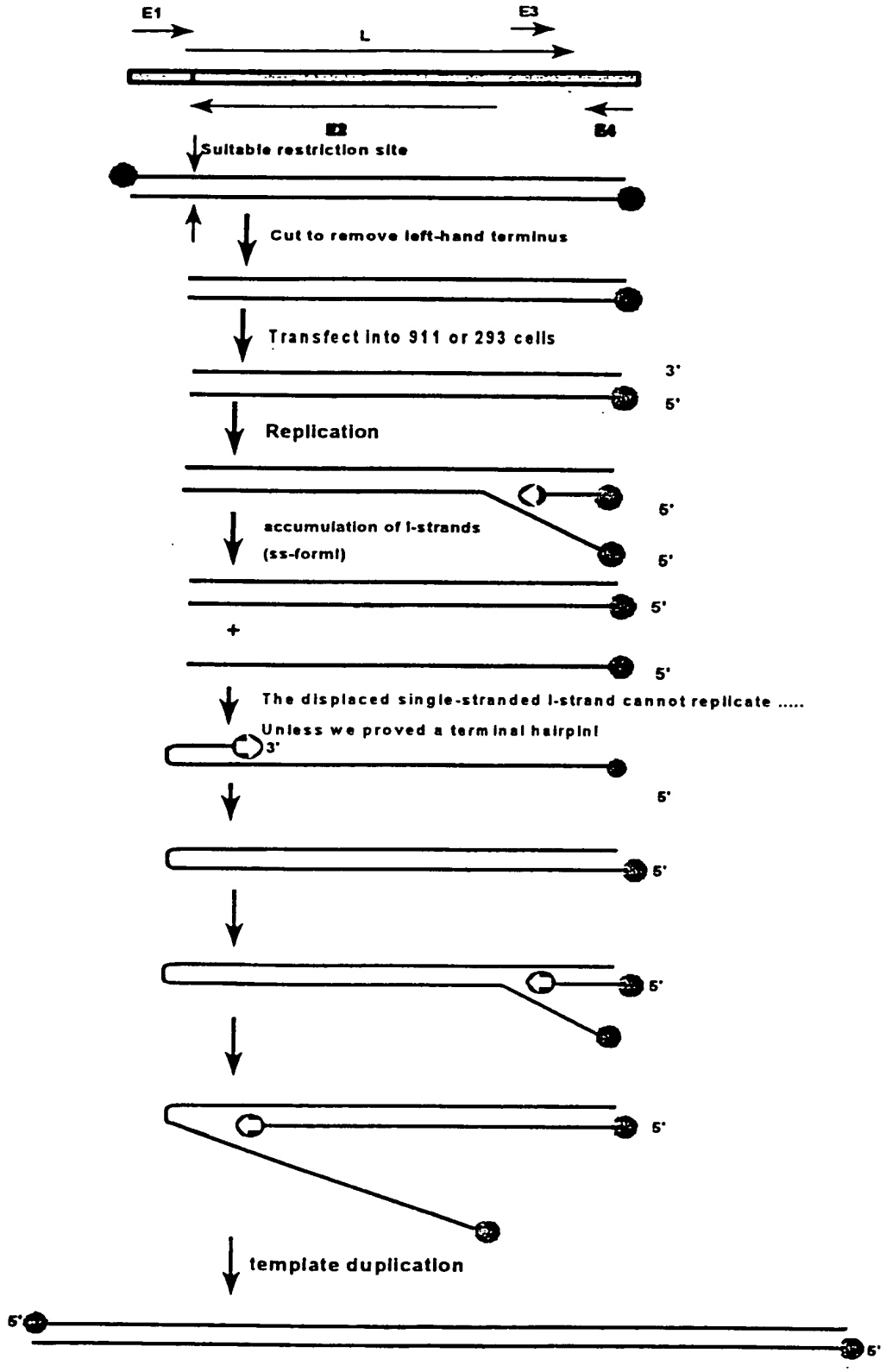


Figure 9


E1
E3
L
E2
E4
Suitable restriction site
Cut to remove left-hand terminus
Transfect into 911 or 293 cells
3'
5'
Replication
5'
accumulation of I-strands
(ss-form)
5'
5'
+
5'
The displaced single-stranded I-strand cannot replicate .....
Unless we proved a terminal hairpin
3'
5'
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template duplication
5'
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Figure 10

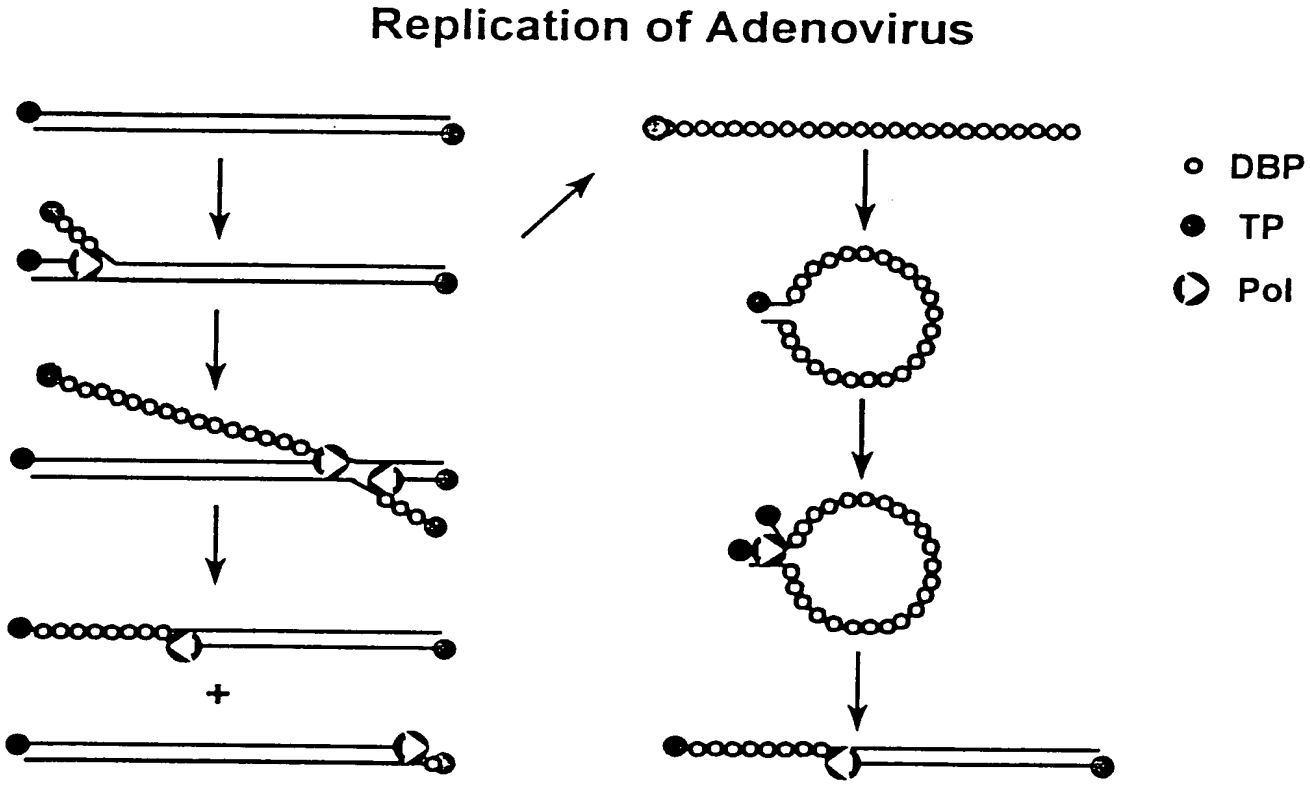


Figure 11

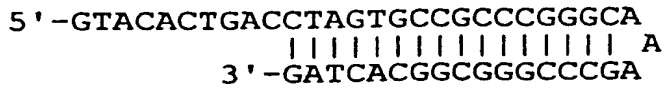
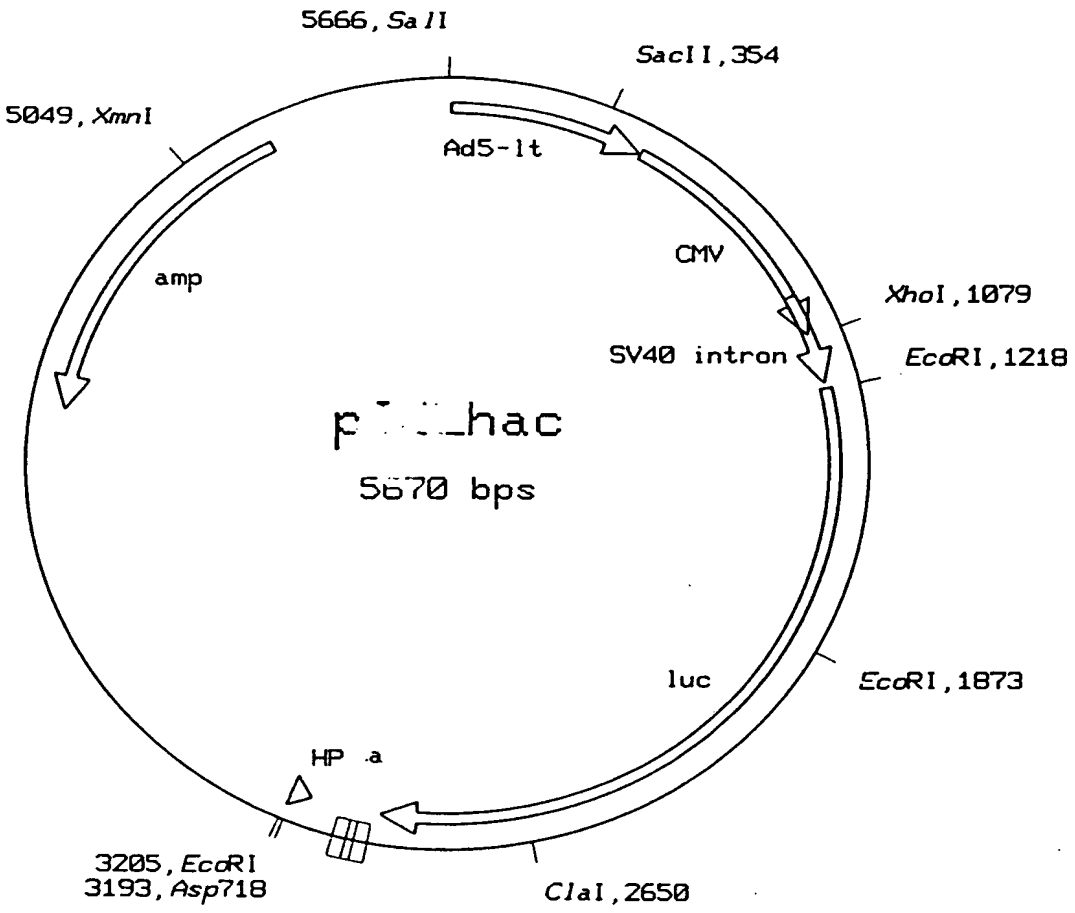


Fig.12. The potential hairpin conformation of a single-stranded DNA molecule that contains the HP/asp sequences used in these studies. Restriction with the restriction endonuclease *Asp*718I of plasmid pICLha$_c$, containing the annealed oligonucleotide pair HP/asp1 en HP/asp2 will yield a linear double-stranded DNA fragment. In cells in which the required adenovirus genes are present, replication can initiate at the terminus that contains the ITR sequence. During the chain elongation, the one of the strands will be displaced. The terminus of the single-stranded displaced- strand molecule can adopt the conformation depicted above. In this conformation the free 3'-terminus can serve as a primer for the cellular and/or adenovirus DNA polymerase, resulting in conversion of the displaced strand in a double-stranded form.

Figure 12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SacII, 354
5049, XmnI
Ad5-lt
CMV
amp
XhoI, 1079
SV40 intron
EcoRI, 1218
p[illegible]hac
5670 bps
luc
EcoRI, 1873
HP a
3205, EcoRI
3193, Asp718
ClaI, 2650

Figure 13

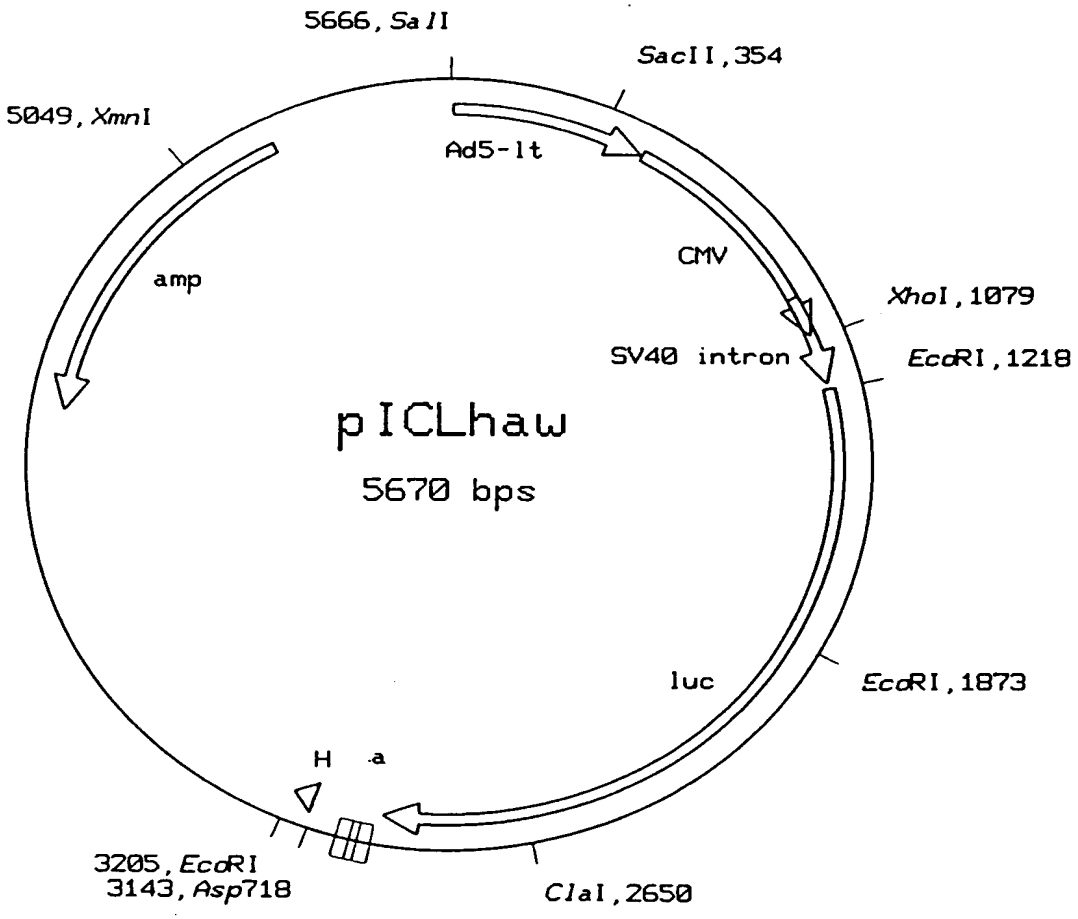
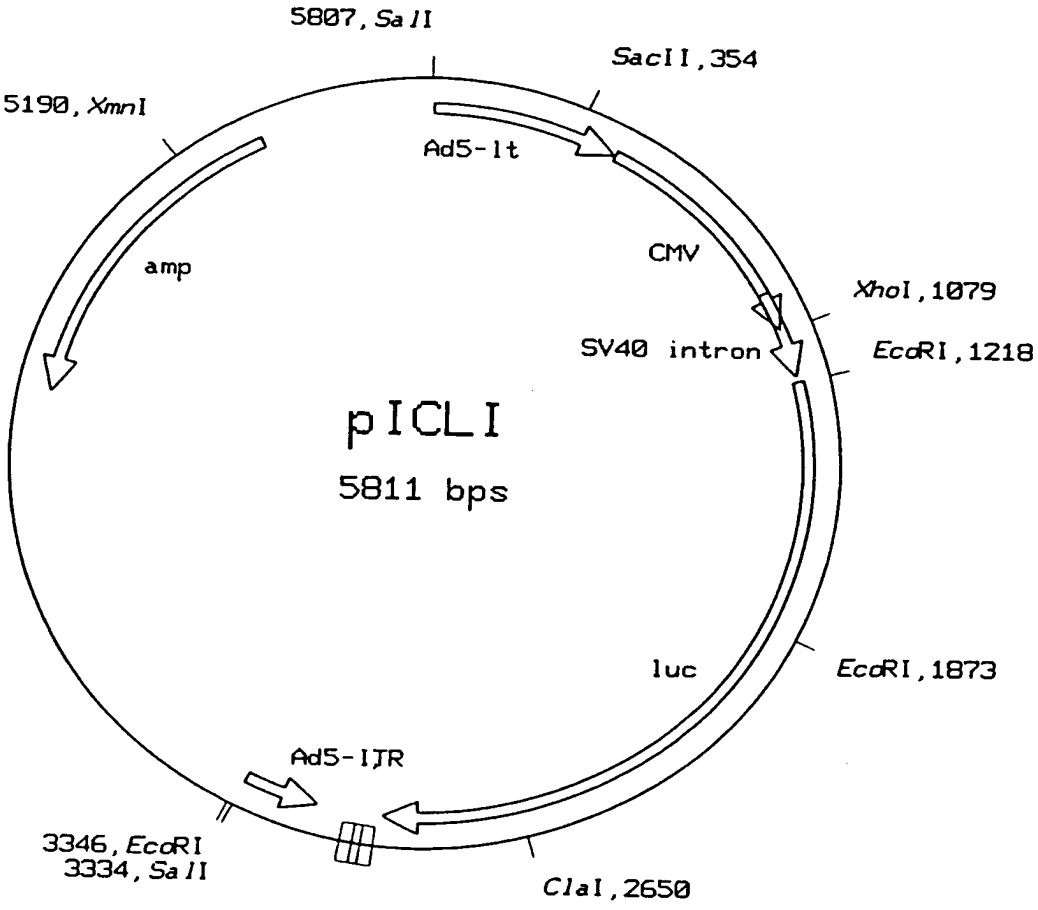

5666, SalI
SacII, 354
5049, XmnI
Ad5-1t
CMV
amp
XhoI, 1079
SV40 intron
EcoRI, 1218
pICLhaw
5670 bps
luc
EcoRI, 1873
H a
3205, EcoRI
3143, Asp718
ClaI, 2650

Figure 14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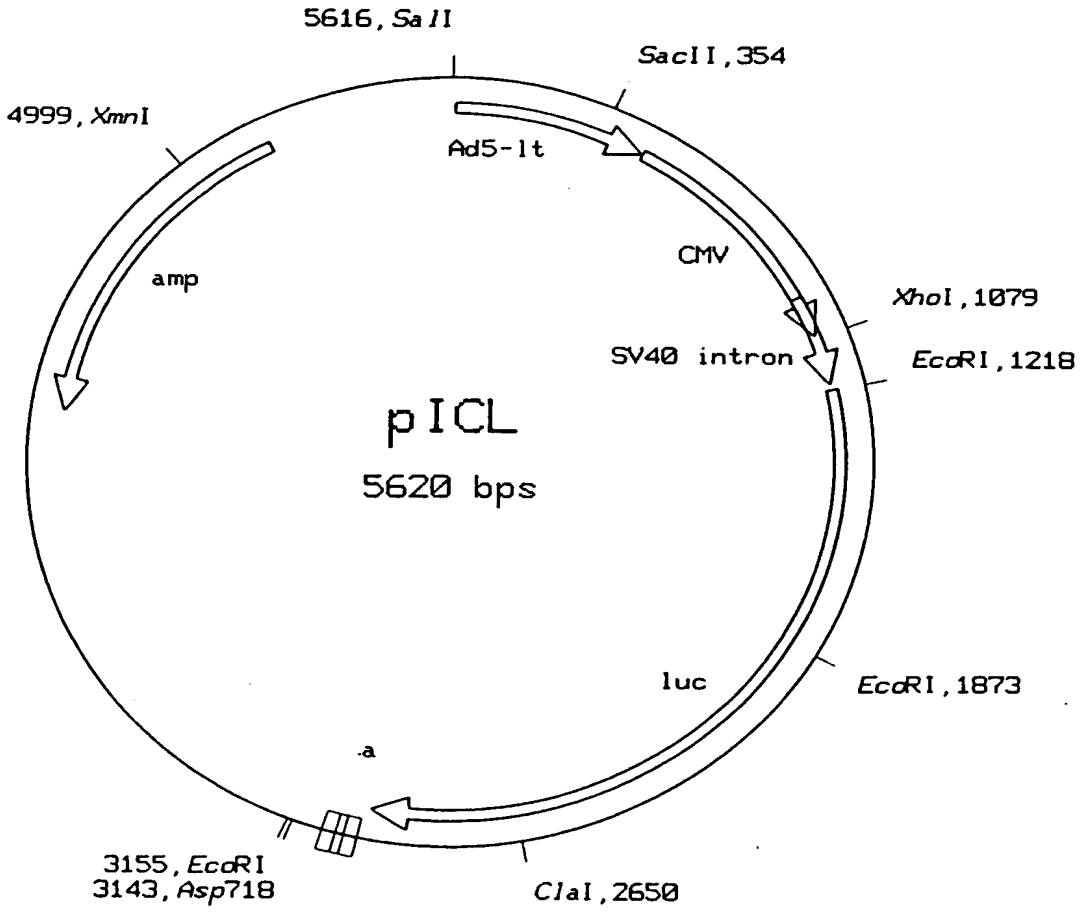
SacII, 354
5190, XmnI
Ad5-1t
amp
CMV
XhoI, 1079
SV40 intron
EcoRI, 1218
pICLI
5811 bps
luc
EcoRI, 1873
Ad5-ITR
3346, EcoRI
3334, SalI
ClaI, 2650

Figure 15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SacII, 354
4999, XmnI
Ad5-1t
CMV
amp
XhoI, 1079
SV40 intron
EcoRI, 1218
pICL
5620 bps
luc
EcoRI, 1873
a
3155, EcoRI
3143, Asp718
ClaI, 2650

Figure 16

Figure 17

Plasmid pICL is derived from the following plasmids:

nt.1 - 457 pMLP10 (Levrerno et al.,)
nt.458 - 1218 pCMVß (Clontech, EMBL Bank no. U02451)
nt.1219 - 3016 pMLP.luc (Introgene, unpublished)
nt.3017 - 5620 pBLCAT5 (Stein et al., 1989)

The plasmid has been constructed as follows:

The tet gene of plasmid pMLP10 has been inactivated by deletion of the BamHI-SalI fragment, to generate pMLP10ΔSB. Using primer ser PCR/MLP1 and PCR/MLP3 a 210 bp fragment containing the Ad5-ITR, flanked by a synthetic SalI restriction site was amplified using pMLP10 DNA as the template. The PCR product was digested with the enzymes EcoRI and SgrAI to generate a 196 bp. fragment. Plasmid pMLP10ΔSB was digested with EcoRI and SgrAI to remove the ITR. This fragment was replaced by the EcoRI-SgrAI-treated PCR fragment to generate pMLP/SAL.

Plasmid pCMV-Luc was digested with PvuII to completion and recirculated to remove the SV40-derived poly-adenylation signal and Ad5 sequences with exception of the Ad5 left-terminus. In the resulting plasmid, pCMV-lucΔAd, the Ad5 ITR was replaced by the Sal-site-flanked ITR from plasmid pMLP/SAL by exchanging the xmnI-SacII fragments. The resulting plasmid, pCMV-lucΔAd/SAL, the Ad5 left terminus and the CMV-driven luciferase gene were isolated as an SalI-SmaI fragment and inserted in the SalI and HpaI digested plasmid pBLCAT5, to form plasmid pICL. Plasmid pICL is represented in figure 15

Plasmid pICL contains the following features:

nt. 1-457 Ad5 left terminus (Sequence 1-457 of human adenovirus type 5)
nt. 458-969 Human cytomegalovirus enhancer and immediate early promoter (Bosthart et al., 1985; from plasmid pCMVß)
nt. 970-1204SV40 19S exon and truncated 16/19S intron (from plasmid pCMVß)
nt. 1218-2987 Firefly luciferase gene (from pMLP.luc)
nt. 3018-3131SV40 tandem poly-adenylation signals from late transcript, derived from plasmid pBLCAT5)
nt. 3132-5620 pUC12 backbone (derived from plasmid pBLCAT5)
nt. 4337-5191ß-lactamase gene (Amp-resistence gene, reverse orientation)

NAME: pICL 5620 BPS DNA CIRCULAR UPDATED 5/01/95
DESCRIPTION: 1 x Ad5-ITR, CMV-luciferase, minimal vector
SEQUENCE: sequence based on the on available information;
Constructions verified by restriction enzyme digests;
Sequence of regions derived from amplified DNA verified by sequence analyses

* * * S E Q U E N C E * * *

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   1  CATCATCAAT AATATACCTT ATTTTGGATT GAAGCCAATA TGATAATGAG GGGGTGGAGT
  61  TTGTGACGTG GCGCGGGGCG TGGGAACGGG GCGGGTGACG TAGTAGTGTG GCGGAAGTGT
 121  GATGTTGCAA GTGTGGCGGA ACACATGTAA GCGACGGATG TGGCAAAAGT GACGTTTTTG
 181  GTGTGCGCCG GTGTACACAG GAAGTGACAA TTTTCGCGCG GTTTTAGGCG GATGTTGTAG
 241  TAAATTTGGG CGTAACCGAG TAAGATTTGG CCATTTTCGC GGGAAAACTG AATAAGAGGA
 301  AGTGAAATCT GAATAATTTT GTGTTACTCA TAGCGCGTAA TATTTGTCTA GGGCCGCGGG
 361  GACTTTGACC GTTTACGTGG AGACTCGCCC AGGTGTTTTT CTCAGGTGTT TTCCGCGTTC
 421  CGGGTCAAAG TTGGCGTTTT ATTATTATAG TCAGGGGCTG CAGGTCGTTA CATAACTTAC
 481  GGTAAATGGC CCGCCTGGCT GACCGCCCAA CGACCCCCGC CCATTGACGT CAATAATGAC
 541  GTATGTTCCC ATAGTAACGC CAATAGGGAC TTTCCATTGA CGTCAATGGG TGGAGTATTT
 601  ACGGTAAACT GCCCACTTGG CAGTACATCA AGTGTATCAT ATGCCAAGTA CGCCCCCTAT
 661  TGACGTCAAT GACGGTAAAT GGCCCGCCTG GCATTATGCC CAGTACATGA CCTTATGGGA
 721  CTTTCCTACT TGGCAGTACA TCTACGTATT AGTCATCGCT ATTACCATGG TGATGCGGTT
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 841  CCCCATTGAC GTCAATGGGA GTTTGTTTTG GCACCAAAAT CAACGGGACT TTCCAAAATG
 901  TCGTAACAAC TCCGCCCCAT TGACGCAAAT GGGCGGTAGG CGTGTACGGT GGGAGGTCTA
 961  TATAAGCAGA GCTCGTTTAG TGAACCGTCA GATCGCCTGG AGACGCCATC CACGCTGTTT
1021  TGACCTCCAT AGAAGACACC GGGACCGATC CAGCCTCCGG ACTCTAGAGG ATCCGGTACT
1081  CGAGGAACTG AAAAACCAGA AAGTTAACTG GTAAGTTTAG TCTTTTTGTC TTTTATTTCA
1141  GGTCCCGGAT CCGGTGGTGG TGCAAATCAA AGAACTGCTC CTCAGTGGAT GTTGCCTTTA
1201  CTTCTAGTAT CAAGCTTGAA TTCCTTTGTG TTACATTCTT GAATGTCGCT CGCAGTGACA
1261  TTAGCATTCC GGTACTGTTG GTAAAATGGA AGACGCCAAA AACATAAAGA AAGGCCCGGC
1321  GCCATTCTAT CCTCTAGAGG ATGGAACCGC TGGAGAGCAA CTGCATAAGG CTATGAAGAG
1381  ATACGCCCTG GTTCCTGGAA CAATTGCTTT TACAGATGCA CATATCGAGG TGAACATCAC
1441  GTACGCGGAA TACTTCGAAA TGTCCGTTCG GTTGGCAGAA GCTATGAAAC GATATGGGCT
1501  GAATACAAAT CACAGAATCG TCGTATGCAG TGAAAACTCT CTTCAATTCT TTATGCCGGT
1561  GTTGGGCGCG TTATTTATCG GAGTTGCAGT TGCGCCCGCG AACGACATTT ATAATGAACG
1621  TGAATTGCTC AACAGTATGA ACATTTCGCA GCCTACCGTA GTGTTTGTTT CCAAAAAGGG
1681  GTTGCAAAAA ATTTTGAACG TGCAAAAAAA ATTACCAATA ATCCAGAAAA TTATTATCAT
1741  GGATTCTAAA ACGGATTACC AGGGATTTCA GTCGATGTAC ACGTTCGTCA CATCTCATCT
1801  ACCTCCCGGT TTTAATGAAT ACGATTTTGT ACCAGAGTCC TTTGATCGTG ACAAAACAAT
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1921  TAGAACTGCC TGCGTCAGAT TCTCGCATGC CAGAGATCCT ATTTTTGGCA ATCAAATCAT
1981  TCCGGATACT GCGATTTTAA GTGTTGTTCC ATTCCATCAC GGTTTTGGAA TGTTTACTAC
2041  ACTCGGATAT TTGATATGTG GATTTCGAGT CGTCTTAATG TATAGATTTG AAGAAGAGCT
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2221  TGCTTCTGGG GGCGCACCTC TTTCGAAAGA AGTCGGGGAA GCGGTTGCAA AACGCTTCCA
2281  TCTTCCAGGG ATACGACAAG GATATGGGCT CACTGAGACT ACATCAGCTA TTCTGATTAC
2341  ACCCGAGGGG GATGATAAAC CGGGCGCGGT CGGTAAAGTT GTTCCATTTT TTGAAGCGAA
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2461  CAGAGGACCT ATGATTATGT CCGGTTATGT AAACAATCCG GAAGCGACCA ACGCCTTGAT
2521  TGACAAGGAT GGATGGCTAC ATTCTGGAGA CATAGCTTAC TGGGACGAAG ACGAACACTT
2581  CTTCATAGTT GACCGCTTGA AGTCTTTAAT TAAATACAAA GGATATCAGG TGGCCCCCGC
2641  TGAATTGGAA TCGATATTGT TACAACACCC CAACATCTTC GACGCGGGCG TGGCAGGTCT
2701  TCCCGACGAT GACGCCGGTG AACTTCCCGC CGCCGTTGTT GTTTTGGAGC ACGGAAAGAC
2761  GATGACGGAA AAAGAGATCG TGGATTACGT CGCCAGTCAA GTAACAACCG CGAAAAAGTT
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2941  TGTAACTGTA TTCAGCGATG ACGAAATTCT TAGCTATTGT AATGGGGGAT CCCCAACTTG
3001  TTTATTGCAG CTTATAATGG TTACAAATAA AGCAATAGCA TCACAAATTT CACAAATAAA
3061  GCATTTTTTT CACTGCATTC TAGTTGTGGT TTGTCCAAAC TCATCAATGT ATCTTATCAT
3121  GTCTGGATCG GATCGATCCC CGGGTACCGA GCTCGAATTC GTAATCATGG TCATAGCTGT
3181  TTCCTGTGTG AAATTGTTAT CCGCTCACAA TTCCACACAA CATACGAGCC GGAAGCATAA
3241  AGTGTAAAGC CTGGGGTGCC TAATGAGTGA GCTAACTCAC ATTAATTGCG TTGCGCTCAC
3301  TGCCCGCTTT CCAGTCGGGA AACCTGTCGT GCCAGCTGCA TTAATGAATC GGCCAACGCG
3361  CGGGGAGAGG CGGTTTGCGT ATTGGGCGCT CTTCCGCTTC CTCGCTCACT GACTCGCTGC
3421  GCTCGGTCGT TCGGCTGCGG CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGGTTAT
3481  CCACAGAATC AGGGGATAAC GCAGGAAAGA ACATGTGAGC AAAAGGCCAG CAAAAGGCCA
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4261  AGATCCTTTT AAATTAAAAA TGAAGTTTTA AATCAATCTA AAGTATATAT GAGTAAACTT
4321  GGTCTGACAG TTACCAATGC TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC
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4501  CAGCAATAAA CCAGCCAGCC GGAAGGGCCG AGCGCAGAAG TGGTCCTGCA ACTTTATCCG
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